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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled THE INHIBITORY NEURAL CONTROL OF GALLBLADDER MOTILITY submitted by PETER M. NAUGHTON in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE IN EXPERIMENTAL SURGERY.

DEDICATION

TO VERONIE AND OUR

CHILDREN PETER AND MARTIN

A B S T R A C T

The periodic storage of bile in the gallbladder has a regulatory effect on the enterohepatic circulation of bile salts. It also appears to produce a condition of stasis which could be important in the formation of cholesterol gallstones. Very little is known about the control of gallbladder motility during its storage phase, but it is evident that inhibitory mechanisms must be involved, as filling of the gallbladder is accompanied by a disproportionately small rise in pressure.

The gallbladder has a cholinergic and alpha-adrenergic stimulatory and beta-adrenergic inhibitory nerve supply, but none of these appear to be the mediator of relaxation during fasting periods. However, the process of receptive relaxation appears to be neuronally mediated. The purpose of this study was to determine if the gallbladder has a nonadrenergic noncholinergic inhibitory nerve supply that could mediate this process. Furthermore, to determine if the neurotransmitter was possibly a purine compound. Intact and isolated strip preparations of guinea-pig and canine gallbladder and canine cystic duct were suspended in an organ bath and connected to a force-displacement transducer, and contractile and inhibitory activities were recorded isometrically with a Grass recorder.

Two series of experiments were performed:

1. Assessment of the effects of purine compounds, adenosine and adenosine triphosphate, on the preparations in the presence and absence of nucleoside transport inhibitors and purine receptor antagonists.

2. Evaluation of the effects of the same agents on the preparations' contractile responses, before and during electrical stimulation, after blocking the sympathetic and parasympathetic nerve supply.

The findings were as follows:

1. A quantifiable relaxant response to adenosine and a dual response of adenosine triphosphate were demonstrated in both species. It was established that these purine compounds act at different receptor sites in the organs studied.

2. The guinea-pig and dog gallbladder and canine cystic duct have a nonadrenergic noncholinergic inhibitory nerve supply. In view of the findings, the mediator of this response is unlikely to be a purine compound.

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INTRODUCTION

The liver produces bile continuously but the flow of the bile to the intestine undergoes large periodic variations, due to the intermittent storage of bile in the gallbladder. The periodic storage and discharge of bile by the gallbladder could have several important physiological effects and may contribute to gallstone formation in susceptible persons. The most notable effect would be on the enterohepatic circulation of bile salts, which are the major organic constituents of bile.

Bile salts are essential for optimal solubilization and absorption of dietary lipids. During fasting--when, presumably, bile salts are not required in the intestine--between 50 and 75% of the bile secreted by the liver is stored in the gallbladder (1, 2). After meals, when dietary lipids enter the duodenum, at least 50% of the stored bile is discharged into the small intestine (1). The intermittent storage and discharge of bile salts also affects the rate of hepatic synthesis of bile salts and the rate of secretion of bile from the liver. The rate of synthesis of bile salts is regulated by the concentration of bile salts returned to the liver in the portal venous portion of the enterohepatic circulation; i.e., it is regulated by a negative-feedback mechanism. During fasting, when a large proportion of bile salts is sequestered in the gallbladder, the concentration of these salts in the enterohepatic circulation diminishes and the hepatic synthesis of new bile salts increases. The rate of secretion of bile is dependent upon this secretion of bile salts. Hepatocytes also secrete cholesterol, phospholipid, bilirubin and other organic

constituents of bile, but bile salts constitute about 80% of the solid component of bile (3). The secretion of bile salts into the bile canaliculi creates an osmotic gradient, along which water and electrolytes flow passively. Thus the gallbladder, by periodically storing and discharging bile, has far-reaching effects upon bile production and composition. As gallbladder motility is controlled by neurohormonal mechanisms, bile production and composition is at least partly regulated by enteric neural and hormonal systems.

The role of the gallbladder in gallstone formation remains unclear. In the populations of the Western Hemisphere, cholesterol is the major constituent of virtually all gallstones (4). Cholesterol is insoluble in water and is held in solution in the aqueous bile by forming micellar complexes with bile salts and phospholipid (5). The ratio of bile salts to cholesterol is critical for maintenance of this solubility (6), so a relative deficiency of bile salts results in the production of lithogenic bile (7). Reduction of the amount of circulating bile salts during their sequestration in the gallbladder leads to increased hepatic synthesis of bile salts, but the total output of bile salts from the liver (recycled and newly synthesized) at this time is reduced. This decrease does not affect cholesterol secretion, so that during fasting the hepatic bile becomes more lithogenic. Thus this tendency to produce lithogenic bile occurs during the gallbladder's storage phase.

Lithogenic bile does not invariably result in the formation of gallstones (8). Other factors that may be concerned in their formation include physiological stasis of bile within the gallbladder during the

storage phase which could promote gallstone formation from lithogenic bile. Also, glycoproteins and cellular debris in the gallbladder could provide nucleation sites for cholesterol during prolonged periods of storage (9). Therefore, from a physiological and pathological point of view the storage phase of gallbladder motility has considerable importance. Until recently, it was assumed that gallbladder motility was inhibited during periods of fasting. In the 1970s it was found that migrating motor complexes sweep through the intestine periodically during fasting. Vantrappen, Peeters, and Janssens (10) showed that there was an increased discharge of bile acids into the duodenum during the passage of these interdigestive motor complexes. The possibility that this was due to contraction of the gallbladder was confirmed by Itoh and Takahashi (11). Thus, at the present time it appears that the gallbladder is relaxed but also contracts periodically during fasting periods. Little is known about the inhibitory control mechanisms which operate during fasting periods.

Although the present study was designed to investigate, specifically, nonadrenergic inhibitory neural control of motility, it has been viewed against the background knowledge of other factors that affect gallbladder motility.

BILIARY MOTILITY

There appears to be a reciprocal relationship between the motility of the gallbladder and of the sphincter of Oddi (12). After meals, when the gallbladder contracts the sphincter of Oddi relaxes. During fasting periods there appears to be relaxation of the gallbladder and contraction

of the sphincter of Oddi. This reciprocal relationship facilitates the discharge of bile into the duodenum after contraction of the gallbladder, and is almost certainly under neurohormonal control. Cholecystokinin, which is primarily responsible for the contraction of the gallbladder in response to meals, has been shown in cats (13) and in dogs and rabbits (14) to produce relaxation of the sphincter. Recent studies have shown that the sphincter relaxes during electrical and mechanical stimulation of the gallbladder and that this reflex is relayed via the celiac plexus (15). On reviewing the literature one gets the impression that hormonal agents are the most important determinants of gallbladder motility and that neural mechanisms play a minor role (16, 17). As quite a lot is known about postprandial biliary motility consequent upon the release of hormonal agents, the effects of hormones on gallbladder motility will be described first.

HORMONAL CONTROL

Cholecystokinin (CCK)--Ivy and Oldberg (18) proposed the name cholecystokinin for the active component of an extract of small-intestine mucosa which, when released in response to the ingestion of food, contracts the gallbladder. Components of the gastric effluent that individually have been shown to promote the flow of bile from the biliary tract into the intestine include hydrochloric acid (19-22), amino acids (23, 24), and fats (25-27). CCK is believed to act directly on the muscle: its action is not abolished by atropine (28), which blocks the parasympathetic nervous system, by drugs that block the alpha- and beta-adrenergic receptors (28), or by tetrodotoxin (29), which blocks all neuronal transmission. This

cholecystokinetic effect arises from the C-terminal portion of the molecule. It has now been shown that CCK exists in different sizes in various tissue extracts. The octapeptide form, which has been isolated in sheep (30), was shown to be, on a molar basis, several times stronger in terms of its activity than the whole molecule (31).

CCK has been shown to induce contraction of the isolated gallbladder of dogs (32), rabbits (33, 34), guinea pigs (35, 36) and humans (37) in vitro. CCK raises the intraluminal pressure of the gallbladder in anaesthetized dogs (38), cats (38), sheep (40), and humans (41) in vivo.

Gastrin--In vitro, both gastrin I and II have been shown to contract the isolated gallbladder of dogs (28) and guinea pigs (42). In vivo, gastrin increases the intraluminal pressure of the gallbladder in dogs (43) and is presumed to have the same effect in other species. A more significant action of gastrin may be its ability to inhibit CCK-induced responses, albeit noncompetitively (44). These actions of gastrin are not surprising, in view of the close similarity of the C-terminal amino acid groups in this hormone and CCK.

Secretin, Glucagon, and Vasactive Intestinal Polypeptide (VIP)--The peptide hormones secretin, glucagon, and VIP are of very similar structure (45). Secretin has a weak effect on the gallbladder; it has been reported to both contract (45) and relax (44) the muscle. Glucagon relaxes preparations of canine gallbladder in vitro, but this effect has not been demonstrated in vivo (46, 47).

VIP has been shown in vitro to both relax the gallbladder (48) and

inhibit the action of CCK on this muscle (45). VIP, originally isolated and chemically characterized from the upper small intestine, has now been shown to occur throughout the gastrointestinal tract (49). It has also been discovered in the central and peripheral nervous system (50), and in the nerves supplying the gallbladder (51). As a result of its widespread distribution it may act as a local hormone.

These hormonal agents are considered to be much more important than neural mechanisms in the post-prandial control of biliary motility (16, 17). Yet the pattern of innervation and the type of responses to neural stimulation appear to be the same in the gallbladder as in other parts of the gastrointestinal tract. Indeed, it is almost certain that interactions between neural and hormonal effects on the gallbladder are functionally of much greater importance than the individual effects of either.

NEURAL CONTROL

The intramural nerve plexus in the gallbladder is similar in composition and distribution to that in the gastrointestinal tract. Sutherland (52), from a study performed on guinea pigs and monkeys, divided the intramural nerve plexus into two main parts--the myenteric plexus and the submucous plexus.

Myenteric Plexus--Preganglionic vagal nerve fibers and postganglionic sympathetic fibers that have passed via the splanchnic nerves and celiac plexus continue along the course of the cystic duct to reach the gallbladder. At their point of entry into the gallbladder, the vagal fibers divide to form a loosely meshed plexus. This plexus, which contains ganglia, lies

between the subserous and muscular layers; it further divides into secondary and tertiary nets, the finest fibers of which form the deep intramuscular plexus. It is believed that postganglionic sympathetic fibers also participate in this plexus (53).

Submucous plexus--This plexus is similar to the myenteric plexus but has a finer structure and smaller ganglia. Communicating rami connect this plexus to the myenteric plexus.

It is apparent that these two plexuses form an extensive network throughout the entire thickness of the gallbladder wall.

Vagal stimulation contracts the gallbladder of the anaesthetized guinea pig (54) and dog (55), and increases the intraluminal pressure of this organ in anaesthetized cats (56). Conversely, after truncal vagotomy the resting pressure within the gallbladder of anaesthetized cats is reduced (57), and dilation of the gallbladder of anaesthetized dogs (58, 59) and humans has been reported.

The gallbladder receives a sympathetic nerve supply; the effect of stimulation from this source depends upon the type of receptor stimulated. Both alpha- and beta-adrenergic receptors have been demonstrated in the gallbladder muscle in cats (60), rabbits (61), and guinea pigs (62). Stimulation of the alpha-adrenergic receptors mediates contractions and of the beta-adrenergic receptors mediates relaxation in these species (60-62).

It has been suggested that peptidergic neurones in the enteric nervous system (ENS) may release VIP, which is known to have direct

effects on the gallbladder (45, 48). In studies with immunocytochemical techniques, VIP has been identified in nerve terminals in the ENS (49) and in the nerves supplying gallbladder (51).

Davison et al. (63) reported that the guinea pig gallbladder has an inhibitory nerve supply that is not adrenergic. They labeled it purinergic, because the effects of adenosine triphosphate mimicked the responses evoked by electrical stimulation of these nerves.

The mechanism of control of biliary motility during storage is not as clear. But gallbladder motility during this period may be very important, because--as already outlined--it is during this time that gallstone formation is likely to occur. It has been known for many years that even very prolonged ingestion of food does not result in complete emptying of the gallbladder (64). There is considerable evidence that the inhibition of cholocystokinesis is an active process; also, it seems unlikely that the gallbladder, which has an active role in emptying, would have only a passive role in filling. One of the proposed functions of inhibition of the discharge of bile is to permit filling of the gallbladder during storage periods.

Receptive relaxation has been demonstrated in other smooth-muscle organs, including the urinary bladder, stomach (65), and esophagus (66, 67). It is conceivable that receptive relaxation could occur during both the filling and the storage phase of gallbladder activity. Ryan and Cohen (44) showed that increase in the volume of the opossum gallbladder was accompanied by a smaller rise in intraluminal pressure than expected, and recently it was shown in baboons that the gallbladder pressure remained

constant over 2-4 hours despite large fluctuations in volume (68).

The most likely mechanism is that receptive relaxation is neuronally mediated. Sympathetic stimulation or an alteration in the balance between parasympathetic (excitatory) and sympathetic (inhibitory) tone could be involved. But stimulation of the splanchnic nerve in cats does not alter the resting gallbladder pressure (69). In addition, receptive relaxation has been demonstrated in the presence of adrenergic blocking agents (70).

Alternatively, this receptive relaxation could be due to stimulation of nonadrenergic, noncholinergic inhibitory nerves. These inhibitory nerves have been demonstrated in the esophagus (66, 67) and stomach (65, 71) and are reported to mediate receptive relaxation in both of these organs. Nonadrenergic inhibitory neurones in the stomach wall are excited by distension of that organ. They then not only elicit muscular hyperpolarization locally but also inhibit intramural excitatory neurones in the same area (71). In this way they mediate receptive relaxation in the stomach. Al-Hassani and Davison (70) have shown in studies of guinea-pig gallbladder preparations that these nonadrenergic nerves are active and that they help to accommodate the stretching of gallbladder muscle.

NONADRENERGIC NONCHOLINERGIC INHIBITORY NERVES--In 1898 Langley (72) noted that stimulation of the vagus nerve relaxed the stomach when the excitatory fibers were blocked with atropine, and similar responses to parasympathetic-nerve stimulation were demonstrated by McSwiney and Robson (73) and Ambache (74). At that time it was assumed that these inhibitory responses were due to stimulation of adrenergic fibers in the parasympathetic nerves. When, in 1963, Burnstock and his colleagues (75) reported inhibitory

responses to electrical field stimulation of isolated smooth muscle of the tenia, the objection was raised that inhibitory junctional potentials recorded during transmural stimulation could be due to a direct muscle effect (76). However, this objection was withdrawn when it was shown (77) that these responses were abolished by tetrodotoxin, which blocks the initiation of action potentials in neuronal tissue but not in smooth muscle. Martinson, in 1965 (78), and Burnstock, Campbell, and Rand in 1966 (79), showed that when cholinergic excitatory innervation is blocked, stimulation of vagal or sacral nerves provokes an inhibitory response of the gut that does not arise from adrenergic fibers. These nonadrenergic inhibitory responses differ in many ways from adrenergic inhibitory responses:

a) The nonadrenergic inhibitory response is elicited at lower frequencies of stimulation than are required to relax the gut by stimulating adrenergic nerves (79).
b) Adrenergic neuron-blocking drugs, or a combination of alpha- and beta-adrenoceptor antagonists, do not inhibit nonadrenergic-induced relaxation (78).
c) Stimulation of intestinal explants grown in organotypic tissue culture elicits a relaxant response after the adrenergic fibers have degenerated (80).
d) During ontogeny, inhibitory responses can be elicited before the adrenergic nerves have reached the gut.

Thus the gut wall contains neural structures that can inhibit muscular activity when they are stimulated at low frequencies. By contrast, stimulation of the perivascular sympathetic nerve supply to the

gut, even when stimulated repeatedly, only slowly or weakly, polarizes the muscle membrane (81, 82). If both types of nerves were adrenergic, the observed differences in transmission would require the intramural inhibitory nerves to release more transmitter per impulse, or release the transmitter nearer to the muscle's receptive surface, so that a larger concentration of catecholamines would arrive at the receptors. Therefore, the adrenaline-blocking drugs would, under appropriate conditions, be expected to selectively reduce the sympathetic responses without inhibiting the effects of transmural stimulation, even if the inhibitory neurones were adrenergic.

More conclusive evidence that the intramural inhibitory nerves are not adrenergic has been obtained with the histochemical fluorescence technique for identifying catecholamines. If the intramural inhibitory nerves were adrenergic, one would expect this technique to reveal fluorescent neurone somata in the enteric plexus. In fact, the numerous histochemical studies of the adrenergic innervation of the gut (83-85) have resulted in complete agreement that there are no adrenergic ganglia in the gut wall. These findings also exclude the possibility, raised by Bucknell (86), that the inhibitory effects of transmural stimulation during blockage of the adrenergic receptors are mediated by catecholamines released from chromaffin cells, for there are no chromaffin cells within the muscular layers.

It might be argued that the gut's inhibitory responses to transmural stimulation are mediated by sensory neurones in the enteric plexus; i.e., that sensory neurones might conduct action potentials antidromically,

releasing an inhibitory substance from the receptive process as is thought to occur in dorsal-root antidromic vasodilator responses. In studies of tenia coli, stimulation of the nerves in a flap of caecal wall left attached to the longitudinal muscle strip relaxed the tenia after treatment with atropine (79). Like the inhibitory response to transmural stimulation of this muscle, these responses persisted after the adrenergic neurones had been blocked. But, unlike the response to stimulation of the tenia, relaxation caused by stimulation of the flap of caecal wall was reduced after treatment with pentolinium (which blocks the nicotine receptors).

The findings imply that some of the nerve fibers stimulated in the flap of caecal wall are cholinergic, making synaptic connections with intramural inhibitory neurones innervating the muscle of the tenia (79). It is extremely unlikely that cholinergic elements in the gut wall are transmitting this activity to the inhibitory neurones antidromically, as a considerable proportion of the vagal inhibition of the stomach is prevented by nicotinic ganglion-blocking drugs (87), demonstrating that these inhibitory nerves are in fact autonomic nerves.

It must therefore be concluded:

1. That transmission from the cholinergic neurones to the inhibitory intramural neurones is the normal orthodromic direction of conduction; and
2. That the intramural inhibitory neurones are postganglionic neurones in autonomic nerve pathways (i.e., motor neurones).

These studies also demonstrate that although the cell bodies of the nonadrenergic inhibitory nerves lie in the gut wall (79), the neurones at

either end of the gut receive input from the central nervous system via parasympathetic pathways. Stimulation of the vagal nerve produces noncholinergic relaxation and inhibitory junctional potentials in the esophagus (88) and stomach (89). The inhibitory fibers in the terminal rectum are activated by the pelvic nerves (90).

If these innibitory nerves are not adrenergic, and their effects are not due to antidromic stimulation of sensory fibers, it can be concluded that they are motor neurones and that the neurotransmitting agent is not definitely known. Holton and Holton (91) were the first to suggest, in 1954, that ATP may be a neurotransmitter, but it was left to Burnstock (92) to crystallize the available data into the general concept that some neurones ('purinergic') might release a purine compound as a synaptic transmitter.

'PURINERGIC' NERVES--The so-called 'purinergic nerve' hypothesis proposes that ATP or a related purine nucleotide is the neurotransmitter for nonadrenergic inhibitory nerves (79, 93).

To establish ATP as the neurotransmitter would require satisfaction of the following criteria (based on the summary by Eccles, in 1964 [94]):

a) ATP and the enzymes necessary for its formation must be present in the inhibitory nerves;
b) ATP must be released from the nerves when they are stimulated;
c) ATP and the transmitter substance released on nerve stimulation must have the same effect on gastrointestinal muscle;
d) Enzymes that inactivate ATP should occur in tissues; and
e) Drugs that alter nerve-muscle transmission should induce similar

alterations when ATP is applied.

Evidence of ATP release during stimulation of 'purinergic' nerves is as follows:

1. Stimulation of the vagus nerves in guinea pigs and toads resulted in venous efflux of adenosine and inosine from the stomach.
2. Stimulation of the intramural nerves of guinea pig tenia coli that had been incubated with [^{3}H]-adenosine resulted in release of radioactive compounds.

Tissues known to contain nucleoside- and nucleotide-metabolizing enzymes (adenosine kinase and deaminase) and ATPases have been identified in smooth-muscle membranes adjacent to nonadrenergic noncholinergic nerve fibers in the intestine in particular species, and in synaptic clefts in the rat cerebral cortex (95).

The argument for the purinergic nerve hypothesis has been eloquently advanced by Burnstock in his review articles (92, 96). Using an electron microscope, Cook and Burnstock (97) classified neuronal cell bodies into 9 types and axon terminals into 8-10 morphologically distinct subsets. They stated that purinergic nerves are characterized by large opaque vesicles, in contrast to the large granular vesicles seen in adrenergic and cholinergic nerves.

The physiological function of these 'NANCI' nerves in the esophagus (66, 67) and stomach (65, 71) is said to relate to receptive relaxation; in the small intestine (98, 99) and colon (100), they are reported to be involved in descending inhibition during peristalsis. These 'NANCI' nerves have been demonstrated elsewhere, in the urinary bladder, the

pyloric (101), ileocecal (103) and internal anal sphincters, and in the anococcygeus (104, 105) and rectococcygeus muscles. Although ATP has been proposed on good evidence as the neurotransmitter of the nonadrenergic inhibitory neurones that have been called 'purinergic' (Burnstock 1972), (92) it has not been definitely established as the nonadrenergic inhibitory transmitter. Other substances that have been investigated as possible neurotransmitting agents include 5-hydroxytryptamine (106), vaso-active intestinal polypeptide (VIP; [107]), enkephalin (108), somatostatin (109), prostaglandins (110), and 'substance P' (111). All of these agents, with the exception of VIP, have been excluded because they did not fulfill the criteria defined by Eccles (94).

The following approaches can be used to evaluate the nonadrenergic inhibitory nerve supply to the gallbladder:

Electron microscopy (EM)--The essence of identification of autonomic nerves by this means is the hypothesis that all nerve fibers and terminals arising from a particular group of nerve cells (or, more precisely, a particular nerve-cell type) display similar axoplasmic configurations, despite variations in size and shape of the termination. In tissues considered to have a cholinergic and adrenergic nerve supply (i.e., iris, heart), two types of nerve structures have been identified on EM (112): one contained small clear vesicles as the cholinergic component; the other contained numerous small vesicles with granular cores (small granular vesicles) but otherwise were similar to the small clear ones and by default had to be adrenergic. Burnstock (92) has claimed that nerve structures which contain large opaque vesicles are purinergic. However, there is not general agreement on this point, similar structures

having been termed neurosecretory (113) and sensory (114). Large opaque vesicles are now split into two morphologic groups: the 1P-type, with vesicles of 200 µm maximal diameter; and the sP-type, with vesicles of maximal size up to 120-140 µm. The major difficulty in morphologic classification by vesicle content is the tendency to extreme variation in vesicle populations. Classical cholinergic and adrenergic vesicles exist; however, it is very difficult with routine fixation procedures to clearly separate the nerve types.

Histochemistry--With the introduction of the formaldehyde-induced fluorescence technique, catecholamines —the adrenergic neurotransmitters— can now be visualized. No comparable techniques for visualizing cholinergic nerves are now available, but there are methods for detecting cholinesterase (115). Olson, Alund, and Norberg (116) reported that purinergic nerves can be stained specifically with quinacrine and then identified with fluorescence microscopy. However, the ability of quinacrine to stain purinergic nerves may relate to the chemical bonding that occurs between quinacrine and ATP (117); if so, this staining method would be nonspecific, as all tissues contain ATP. Olson, Alund, and Norberg (116) further reported that staining does not occur specifically unless the tissues are kept in a reasonably balanced medium and at body temperature; this suggests that a specific uptake process in the nerve membrane may be involved. It is noteworthy that quinacrine histofluorescence has been demonstrated only in tissues that reportedly have a nonadrenergic inhibitory nerve supply.

Pharmacologic approach--Nerve effector transmission in adrenergic and

cholinergic systems is susceptible to pharmacologic intervention. For example, if it were claimed that the innervation of a particular organ was cholinergic, the minimal pharmacologic evidence to support this would be that acetycholine would mimic the effect of nerve stimulation, that atropine would inhibit transmission, and that an anticholinesterase would enhance the effect of both nerve stimulation and acetylcholine.

In the present study, the pharmacologic approach was applied to evaluate the nonadrenergic inhibitory supply to the gallbladder.

AIMS OF THE PRESENT STUDY

This study was designed to investigate the part played by nonadrenergic noncholinergic nerves in the mediation of gallbladder relaxation.

Answers were sought to the following questions:

(a) What are the effects of purine compounds on the gallbladder?

(b) What are the effects of purine compounds on the gallbladder when known potentiating and antagonistic agents are present?

(c) Does the gallbladder have a nonadrenergic noncholinergic inhibitory nerve supply?

(d) Can the antagonists or potentiators of the purine compounds affect the nerve-mediated inhibitory responses similarly?

The guinea pig and dog were chosen as the experimental animals, two species in which the reported concentrating and contractile abilities of the gallbladder differ (118, 119).

METHODS

ANIMALS

Guinea Pigs--Adult guinea pigs of either gender weighing 350-500 g were employed. No more than six guinea pigs were housed in one cage. One guinea pig was chosen at random on each experimental day.

Dogs--Healthy male or female adult mongrel dogs that had been rendered free of intestinal parasites and had received antirabic and distemper vaccines were used. Their weights ranged from 10-20 kg.

Environmental conditions were kept constant for both groups of animals. The temperature was maintained at 21° C and the lighting cycle was 12 h light and 12 h darkness. All animals were refused food, but not fluids, for 12 h before use.

GALLBLADDER PREPARATIONS

Guinea-pig gallbladder--Guinea pigs were killed by a process of stunning and exsanguination. The abdomen was opened via a midline incision. An artery forceps was applied to the cystic duct near its junction with the common bile duct. The cystic duct was then divided distal to the clamp and the intact gallbladder was gently stripped from the hepatic bed. The gallbladder was placed in Tyrode solution and the cystic duct was cannulated with a metal tube and secured in place with a silk ligature (Fig. 1). The fundus of the gallbladder was connected by means of a 000 silk ligature to an isometric transducer. The gallbladder was suspended in an organ bath (25 ml) containing Tyrode solution which was aerated with carbogen (95% O_2, 5% CO_2).

Guinea-pig gallbladder strips--Gallbladders were excised and placed in carbogenated Krebs' solution, washed free of bile, and divided into four longitudinal strips. One end of each strip was attached by means of a 000 silk ligature to a platinum hook electrode at the bottom of the organ bath; the opposite end of the tissue strip was attached by a 000 silk ligature to an isometric transducer (Fig. 2). In this way the tissues were mounted in an organ bath (5 ml) containing Krebs' solution (95% O_2, 5% CO_2) and maintained at 37° C.

Dog gallbladder strips--Dogs were anaesthetized with an intravenous injection of sodium pentobarbital (300 mg/kg). An endotracheal tube was then inserted to maintain an airway. The animals were allowed to breathe spontaneously (room air) during the surgical procedure. A midline incision

was then performed and the gallbladder and a length of cystic duct were removed. The animals were then sacrificed with an overdose of pentobarbital (800 mg). The gallbladder was washed free of bile in carbogenated Krebs' solution. Strips, cut in a longitudinal direction, were taken from the fundus of the gallbladder. These strips were then suspended in an organ bath and maintained in a similar manner to the guinea-pig strips. Circular strips were taken from the cystic duct in five cases.

Time-dependent changes in the sensitivity of the gallbladder strips both to electrical stimulation and exogenously applied pharmacological agents were demonstrated in initial experiments. Later experiments were carefully controlled to allow for time-dependent changes.

RECORDING TISSUE RESPONSES

The background tension at which both the intact gallbladder and the guinea pig and dog gallbladder strips were set initially was 500 mg. The tissues were allowed to stabilize for 45 min to this tension before the start of each experiment. During the stabilization period the tissues were washed continuously with a preheated Krebs' solution at the rate of 2 ml/min. Tissues were exposed to different concentrations of agonists for one to two minutes or until the maximum response had been obtained. The agonists were then washed from the organ bath by overflow with preheated Krebs' solution before the next concentration of the agonist was added to the bath. A 5-minute waiting period was allowed between drug additions. At the completion of each concentration response curve, the tissue was allowed to recover for 45 min; during this time the tension of

the tissue was readjusted to 500 mg. The tissue was washed by overflow continuously with preheated Krebs' solution during this stage.

ELECTRICAL FIELD STIMULATION

The tissue was suspended in Krebs' solution in the organ bath and current was applied throughout the bath. It is important to place the tissue in the centre of the bath and not near the walls. The electrodes were placed so that a uniform electric field was obtained throughout the bath except for some unavoidable distortion near the chamber walls. In this study the electrical current was applied in two directions: (a) *transversely* - in which the tissue was suspended between two parallel electrodes, and (b) *longitudinally* - in which the electrodes were placed at opposite ends of the bath and tissue was suspended between them. Current in this situation was applied parallel to the long axis of the tissue.

Both sets of electrodes in this study were platinum. The stimulating parameters found to be optimal and used throughout these initial experiments were a pulse width of 1 ms, an amplitude of 100 volts, a stimulation period of 30 s and a frequency range of 0.5-80 Hz supplied by a Grass stimulator (S9). In some dog gallbladder strips the pulse width was reduced to 0.5 ms. A 5-min recovery period was allowed between periods of stimulation.

During the course of an experiment the tissue was washed continuously with preheated Krebs' solution (in this way, any byproducts of stimulation were prevented from accumulating in the organ bath and causing direct

tissue effects). Irrespective of the stimulation frequency used, recovery of the original tone of the tissue was achieved usually within 4 min after discontinuing stimulation. Washing the preparation with preheated Krebs' facilitated this recovery.

Contraction or relaxation of the tissue in response to electrical field stimulation or to drugs was measured as changes in isometric tension with a Grass (FT03C) force-displacement transducer and displayed on a Grass (Model 7D) polygraph.

EVALUATION OF RESULTS

Complete concentration- and frequency–response curves were obtained; responses expressed as changes in tension (mg) were plotted against the log of the concentration or frequency. The maximum inhibitory response that could be obtained was 500 mg, the resting tension at which the tissue was set. In those cases where CCK was added to increase the background tone of the preparation, relaxant responses greater than 500 mg could be obtained.

ED50 value - the concentration of agonist which produces half the maximum response was calculated for each individual concentration–effect curve on each of the strips.

EF50 value - the frequency which produces half the maximum response was calculated for each individual frequency–response curve on each of the strips.

Statistical Analysis of Data--Results are expressed as mean ± standard error of the mean (s.e. mean). Significant levels for the difference between groups were estimated using Student's paired, and in relevant cases, unpaired t test. The difference between groups was judged to be significant when $P < 0.05$.

SOLUTIONS AND DRUGS

Tyrode solution had the following composition (mM):
NaCl, 137; KCl, 2.7; $CaCl_2$, 1.8; $MgCl_2$, 1.05; $NaHCO_3$, 23.1; NaH_2PO_4, 0.42 and glucose, 5.56.

Krebs' solution had the following composition (mM):
NaCl, 116; KCl, 5.4; $CaCl_2$, 2.5; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 22.0 and glucose, 11.2.

Both solutions were made in distilled, deionized water, aerated with carbogen (95% O_2, 5% CO_2) and maintained at 37° C.

The background tone of the tissue was raised in relevant cases with cholecystokinin (CCK, 0.06 Ivy dog units/ml). CCK was added directly to the organ bath, and as soon as the CCK-induced contraction reached a plateau phase (usually within 4-6 min after its addition), the agent to be evaluated could then be added to the organ bath.

The nucleoside transport inhibitor, 6-(2-hydroxy-4-nitrobenzyl)-thioguanosine (HNBTG, 10 μM), was added directly to the organ bath. Tissues were allowed to incubate with HNBTG for 25 min before the response

to any of the purine compounds was assessed. Because of the very high affinity of HNBTG for glass tubing, at the end of each experiment all glassware and organ baths with which it was in contact were cleansed with chromic acid and potassium hydroxide.

When the adenosine-receptor antagonist, theophylline (in the form of aminophylline) was used, the tissue was exposed to this agent for 45 min prior to initiating concentration response studies. Because of the difficulties of getting theophylline into solution, only aminophylline which is $(\text{theophylline})_2$ ethyledinamine was used in this study. Following the addition of the agonist, the tissue was washed twice with Krebs' solution containing aminophylline before the next concentration of the agonist was added.

Indomethacin was dissolved in equimolar Na_2CO_3 solution and immediately neutralized with INHCl and used within 2 h.

Stock solutions (10^{-1}M) of adenosine, 2-chloroadenosine, N^6-(L-phenylisopropyl) adenosine, 5^1-N-ethylcarboxamido adenosine (NECA) were made in dimethylsulfoxide (DMSO) and stored frozen. DMSO was used as a solvent because of the low solubility of the substituted adenosine derivatives in water. DMSO alone, in the highest concentration (1%), did not alter the resting tone of the tissue.

Drugs used and their sources were as follows: adenosine, adenosine deaminase, adenosine triphosphate, aminophylline, atropine sulphate, 2-chloroadenosine, guanethidine, indomethacin, propranolol, tetrodotoxin (Sigma Chemical Company); cholecystokinin (Kabi Vitrum Canada); dimethyl-

sulfoxide (DMSO) (Fisher Scientific Company); kinevac (Squibb Chemical Company); phentolamine mesylate (Ciba); sodium pentobarbital (Diamond Laboratories); 6-(2-hydroxy-4-nitrobenzyl)-thioguanosine (HNBTG) was kindly donated by Dr. A.R.P. Paterson, Cancer Research Unit, University of Alberta.

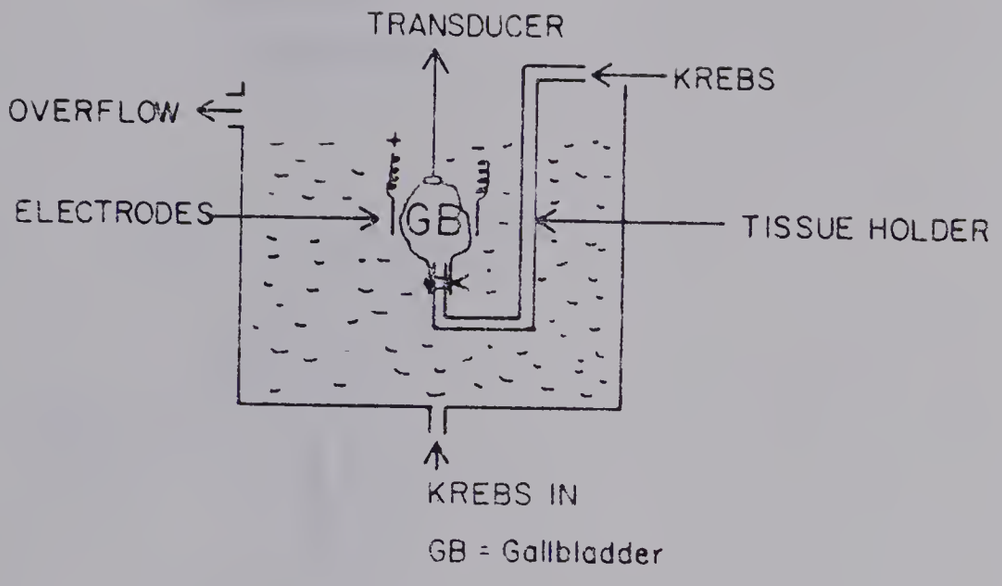

FIGURE 1

Intact gallbladder (GB) preparation cannulated and suspended via a tissue holder in tyrode solution. The gallbladder was connected to an isometric transducer, drugs were applied to the external surface of the gallbladder. Electrical stimuli were applied by platinum electrodes touching the external surface of the gallbladder.

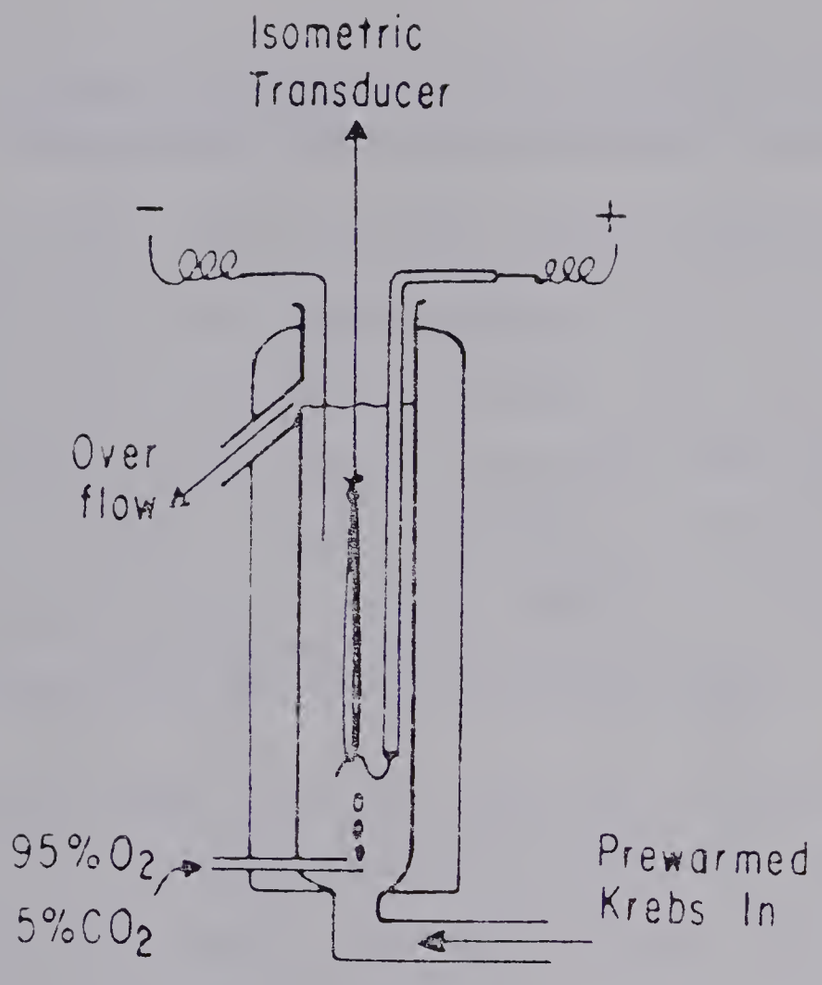

FIGURE 2

Schematic illustration of organ bath in which strip preparations were studied.

RESULTS

Responses of guinea-pig intact gallbladder to exogenously applied pharmacological agents and to electrical stimulation

Acetylcholine ($3X10^{-6}$ *to* 10^{-3}M) produced concentration-dependent contractions of the intact gallbladder (Fig. 3). After a concentration of acetylcholine had reached its maximum effect, the tone of the tissue frequently did not return to its original level despite washing by overflow 2-3 times. Following pretreatment of the preparation with the muscarinic antagonist, atropine ($3X10^{-6}$M), for 45 min, the response to exogenously applied acetylcholine was abolished.

Adenosine ($3X10^{-6}$ *to* 10^{-3}M) consistently relaxed the intact gallbladder (Fig. 4). Following the addition of adenosine, recovery of the original tone of the tissue was slow despite frequent washing by overflow and manually adjusting the tension.

Adenosine Triphosphate (ATP, $3X10^{-6}$ *to* 10^{-3}M) usually contracted the intact gallbladder (Fig. 4). Occasionally a small relaxation preceding a contraction occurred (10%). The onset of the contractile response to ATP was much slower than that of acetylcholine. It was also found during the initial experiments with ATP and adenosine that, following the addition of either to the organ bath, the spontaneous activity of the preparation increased. This occurred irrespective of whether that concentration caused a direct tissue response. In the presence of this marked spontaneous activity the detection of stimulation-induced changes in tone became very

difficult. Pretreatment of the preparation with indomethacin ($3X10^{-6}M$), a prostaglandin sythetase inhibitor (120), which by itself did not significantly alter the resting tone, significantly reduced the degree of spontaneous activity.

Electrical Field Stimulation--Field stimulation of the gallbladder in a transverse direction by means of a pair of electrodes touching the external surface of the gallbladder produced a frequency-dependent contractile response (Fig. 5). The maximum response was seen at a frequency of 30 Hz EF_{50} 4.5 ± 0.5 Hz. After a series of initial experiments, it was found that incubation of the gallbladder with atropine ($3X10^{-6}M$) abolished the responses to field stimulation except at high frequencies (5-30 Hz), where small contractile responses were seen (Fig. 5).

In a significant proportion of experiments (>20%), it was found that during the preparation of a concentration–response curve, the tissue would cease to respond. When the pH of the Tyrode solution was measured at this stage, it was frequently found to be acidotic. Because of this alteration in pH levels with consequent loss of tissue response, and because of excess spontaneous activity, it was decided to continue this study using isolated strips rather than the intact organ. Tyrode solution as the nutrient medium was replaced by Krebs' solution with the strip preparations.

Responses of isolated strips of guinea-pig gallbladder to adenosine, adenosine analogs, and ATP

The responses to adenosine and ATP were assessed under two conditions:

(a) Resting tissue tone of 500 mg

Adenosine ($3X10^{-6}$ to 10^{-3}M) relaxed gallbladder strips (Fig. 6 ED_{50} 70 ± 1.0 μM). A time-dependent change in the response to adenosine, which was statistically significant, occurred in the concentration range of $3X10^{-4}$ to 10^{-3}M (Fig. 6).

The effect of the proposed purine antagonist, aminophylline, on the adenosine-mediated response was evaluated. In an initial set of experiments (n=3), it was found that aminophylline (10^{-5}M) had no direct effect on the tone of the tissue. Following incubation of the tissues with this concentration of aminophylline for 45 min, the adenosine-mediated relaxations were significantly antagonized (Fig. 7).

The effect of the nucleoside transport inhibitor, 6-(2-hydroxy-4-nitrobenzyl)-thioguanosine (HNBTG), was assessed on the adenosine-mediated responses. From an initial series of experiments (n=3) it was found that, at a concentration of 10^{-6}M, HNBTG had no direct effects on the tone of the tissue. Higher concentrations of HNBTG induced relaxation of the tissues. Following incubation of the tissue with HNBTG (10^{-6}M) for 25 min, the relaxant responses to adenosine in the concentration range of $3X10^{-6}$ to 10^{-4}M were significantly potentiated (Fig. 8) (ED_{50} 20 ± 0.5 μM). Aminophylline (10^{-5}M) significantly antagonized the adenosine-mediated

relaxations seen after pretreatment of the tissues with HNBTG (Fig. 8 65 ± 0.7 μM)

(b) Raised Tone

The background tone of the preparations was raised with CCK (0.06 U/ml). This concentration of CCK was chosen because it was shown to produce a submaximal reproducible contraction of the tissue (Fig. 9). Following the addition of CCK, the tissue contracted almost immediately; however it usually took 4-6 min before a plateau contraction was reached. Surprisingly, when the background tone of the preparation was raised with CCK, the amplitude of the relaxant responses to adenosine were similar to those obtained in the absence of CCK (Fig. 6).

(a) Resting tissue tone of 500 mg

Adenosine Triphosphate (ATP) ($3X10^{-6}$ to 10^{-4}M) contracted gallbladder muscle (Fig. 10). In the concentration range of $3X10^{-4}$ to 10^{-3}M, ATP produced contractions in 70% of the strips; in the remainder, either muscle relaxation or a relaxation preceding a contraction occurred. After exposure of the tissues to indomethacin ($3X10^{-6}$M) for 45 min, predominantly muscle relaxation occurred, but in 10% of strips a small contraction occurred.

(b) Tone raised with CCK (0.06 U/ml)

When the background tone of the preparation was raised with CCK (0.06 U/ml), predominantly muscle relaxations occurred after the addition of ATP ($3X10^{-5}$ to 10^{-3}M); with lower concentrations of ATP ($3X10^{-6}$ to 10^{-5}M), muscle contraction occurred in 20% of strips. However, when the background

tone was raised with CCK, and following incubation with indomethacin ($3X10^{-6}M$), only relaxant responses occurred following the addition of ATP (Fig. 11).

ATP-mediated relaxations in the presence of indomethacin and CCK were not significantly antagonized by aminophylline ($10^{-5}M$) except at the single high concentration of ATP ($10^{-3}M$) (Fig. 11). Following incubation of the tissue with the nucleoside transport inhibitor HNBTG, the inhibitory responses to ATP were enhanced only in the concentration range $3X10^{-6}$ to $10^{-5}M$. ATP-mediated relaxations in the presence of HNBTG were inhibited by aminophylline $10^{-5}M$ (Fig. 12). These results are the opposite to those seen with adenosine.

Effect of adenosine deaminase on the ATP- and adenosine-mediated relaxations

The enzyme adenosine deaminase converts adenosine into inosine. Consequently, in the presence of an adequate concentration of this enzyme, one can exclude any actions of ATP as being due to its degradation to adenosine. Adenosine ($10^{-4}M$) or ATP ($10^{-4}M$) was added to the organ bath, the responses were noted, and the tissue was washed twice. Adenosine deaminase (8 i.u/ml, determined after 3 initial experiments) was added directly to the organ bath and allowed to incubate with the tissue for 20 min. The response to adenosine ($10^{-4}M$) or ATP ($10^{-4}M$) was again determined. Only in those cases where adenosine deaminase abolished the response to adenosine were responses to ATP recorded. After incubation with adenosine deaminase, the relaxant response to ATP, while not being abolished, was significantly reduced (Fig. 13).

Adenosine analogs

The response of the tissue to the adenosine analogs 2-chloroadenosine, 5^1-N-ethylcarboxamidoadenosine (NECA), and phenylisopropyladenosine (PIA), were then assessed. These analogs have been reported to be much more potent than adenosine. In this study the response to these analogs was compared with adenosine after incubation of the tissue with HNBTG. All analogs produced concentration-dependent relaxant responses to the gallbladder. All analogs except PIA had the same sensitivity. Significantly, the responses to 2-chloroadenosine and NECA were similar to that of adenosine in the presence of HNBTG (Fig. 14).

Responses of guinea-pig gallbladder strips to electrical (field) stimulation

The response of guinea-pig gallbladder strips to electrical (field) stimulation was characterized by a contraction during the period of stimulation, followed usually by a relaxation at the end of the stimulation period. The contractile response to field stimulation was frequency-dependent, beginning usually at a frequency of 0.5 Hz; the maximum response was obtained at a frequency of 30-50 Hz, EF_{50} (4.5 ± 0.5 Hz). The inhibitory response, seen at the end of the stimulation period, also tended to be frequency-dependent (Fig. 15). Following pretreatment of the tissue with TTX (10^{-6}M) for 20 min, the contractile response to field stimulation was abolished in the lower frequency range (0.5-10 Hz). However, at high frequencies (20-80 Hz) small contractile responses were seen (Fig. 15, 16). Electrical field stimulation following pretreatment of tissues with atropine (10^{-5}M) and guanethidine ($2X10^{-5}$M), concentrations which

would inhibit both parasympathetic-and sympathetic-stimulated responses, resulted in inhibitory responses which were maximal in the lower frequency range of 0.5-10 Hz (Fig. 17, 18). These concentrations of atropine and guanethidine were chosen after it was shown they inhibited cholinergic and adrenergic action. Following pretreatment of the tissues with TTX (10^{-6}M) these inhibitory responses were virtually abolished (Fig. 17).

Pharmacological studies on these nonadrenergic, noncholinergic inhibitory NANCI responses

Following pretreatment of the tissue with the nucleoside transport inhibitor HNBTG (10^{-6}M), there was a statistically significant increase in the amplitude of the inhibitory responses in the frequency range of 0.5-2 Hz (Fig. 19). HNBTG had no effect on the inhibitory responses elicited at higher frequencies. The inhibitory response curve was shifted after pretreatment with aminophylline (10^{-5}M), even though this was not statistically significantly at any of the points tested (Fig. 19).

When the background tone of the preparation was raised with CCK (0.06 u/ml), the amplitude of the NANCI responses was greater than in the tissues in which the background tone was not raised (Fig. 20, 21). The inhibitory responses seen when the tone was increased with CCK were not abolished by TTX (Fig. 20, 21). To exclude the possibility of an impurity in CCK producing these TTX-resistant responses, the octapeptide derivative (Kinevac) was used to raise the tissue tone. Under these conditions, TTX-resistant inhibitory responses were again demonstrated. In addition, when histamine (10^{-8}M) was used to increase the tone of the tissue, TTX-

resistant inhibitory responses were again demonstrated.

Reducing the temperature of the Krebs' solution to 20° C is known to abolish spontaneous activity and block neuronal transmission (121). When the temperature of the gallbladder strips was reduced to 20° C for one hour before the study was commenced and with the background tone raised with CCK, these NANCI responses were abolished (Fig.22). By contrast, the tissue still contracted to CCK or ATP and relaxed in response to adenosine (Fig. 22).

Responses of isolated strips of dog gallbladder to adenosine, ATP, and INA

Adenosine consistently produced relaxation of dog gallbladder strips, irrespective of the degree of resting tone of the preparation (Fig. 23). By contrast, the response obtained with ATP was variable and depended upon the tone of the preparation. In low-tone preparations, a contractile response was frequently seen; however, no attempt was made to quantitate this response, due to its variability. In tissues pretreated with the prostaglandin synthetase inhibitor, indomethacin ($3X10^{-6}M$), and with the tone raised with CCK, inhibitory responses were produced in response to ATP (Fig. 23).

In 6 gallbladders (20 strips), concentration-dependent inhibitory responses to the β-adrenoceptor agonist, isoproterenol (INA), were produced (Fig. 24). Incubation of 10 strips (n=6) with the β-adrenergic blocker, propranolol ($2X10^{-5}M$), for 45 min antagonised the inhibitory responses to INA; small time-dependent changes to INA were seen in the remaining strips (Fig. 24).

Responses of dog gallbladder strips to electrical (field) stimulation

Electrical current was applied in either of two directions:

(a) Longitudinal direction

Field stimulation in a longitudinal direction of isolated strips of dog gallbladder produced frequency-dependent contractile responses (Fig. 25). Incubation of the tissues with atropine (10^{-5}M) and guanethidine ($2X10^{-5}$M) uncovered inhibitory responses in response to field stimulation, which were maximal in the frequency range 5-20 Hz (Fig. 26). These inhibitory responses were not antagonized following pretreatment of the tissue with TTX (10^{-6}M).

(b) Transverse direction

Electrical field stimulation in a transverse direction of isolated strips of gallbladder produced frequency-dependent contractile responses similar to those seen with a longitudinal electric field (Fig. 25). Pretreatment of the tissue with TTX (10^{-6}M) antagonised these responses to field stimulation at the lower frequency range (1-10 Hz). Small contractile responses were seen at higher frequencies (20-80 Hz; Fig. 25). Field stimulation after incubation of the tissues with atropine (10^{-5}M) and guanethidine ($2X10^{-5}$M) uncovered inhibitory responses, which were maximal in the frequency range 1-20 Hz (Fig. 26, 27). These inhibitory responses, while not frequency-dependent, were abolished following pretreatment of the tissue with TTX (10^{-6}M; Fig. 26, 27). Similarly, field stimulation in a transverse direction of circular strips of cystic duct (n=5) after incubation with atropine (10^{-5}M) and guanethidine ($2X10^{-5}$M) produced nonadrenergic, noncholinergic, TTX-sensitive inhibitory responses (Fig. 28).

Effect of variation of the pulse duration of stimulation on the contractile and inhibitory responses with a longitudinal electrical field

TTX-resistant inhibitory responses were seen twice in this study: (a) guinea-pig gallbladder in the presence of CCK, (b) dog gallbladder; the common factor in both situations was a longitudinal electric field. We decided to evaluate the effect of varying the pulse duration of stimulation, also making sure that the tissue was not in contact with the electrodes. Reducing the pulse duration from 1 ms to 0.5 ms had no significant effect on the contractile responses (Fig. 29). However, inhibitory responses (n=5) elicited with a pulse duration of 0.5 ms and with the background tone raised with CCK were abolished following pretreatment of the preparation with TTX (Fig. 30).

Autoinhibition studies with adenosine and ATP

In a further set of experiments (n=5), field stimulation in a transverse direction of tissues treated with a combination of atropine, guanethidine, and indomethacin at a frequency of 5 Hz elicited reproducible inhibitory responses. Autoinhibition was produced by adding ATP (10^{-4}M) or adenosine (10^{-4}M) directly to the organ bath at 1-min intervals until they produced no response (about 5 times). The tissue was not washed between additions, so that these purine compounds were allowed to accumulate in the bath. Electrical stimulation at 5 Hz was then repeated. In ten strips (n=5) inhibitory responses to field stimulation at 5 Hz were not significantly different from the pre-autoinhibition controls, even though the tissue had been completely desensitized to ATP or adenosine (Fig. 31).

Magnitude of nerve-mediated NANCI responses

A histogram comparing the maximum inhibitory responses obtained with ATP, INA, adenosine and the nonadrenergic inhibitory response on field stimulation is shown in Fig. 32. The nonadrenergic inhibitory response, which is very similar to the maximum ATP response, is only 28% of the maximum response obtained with INA.

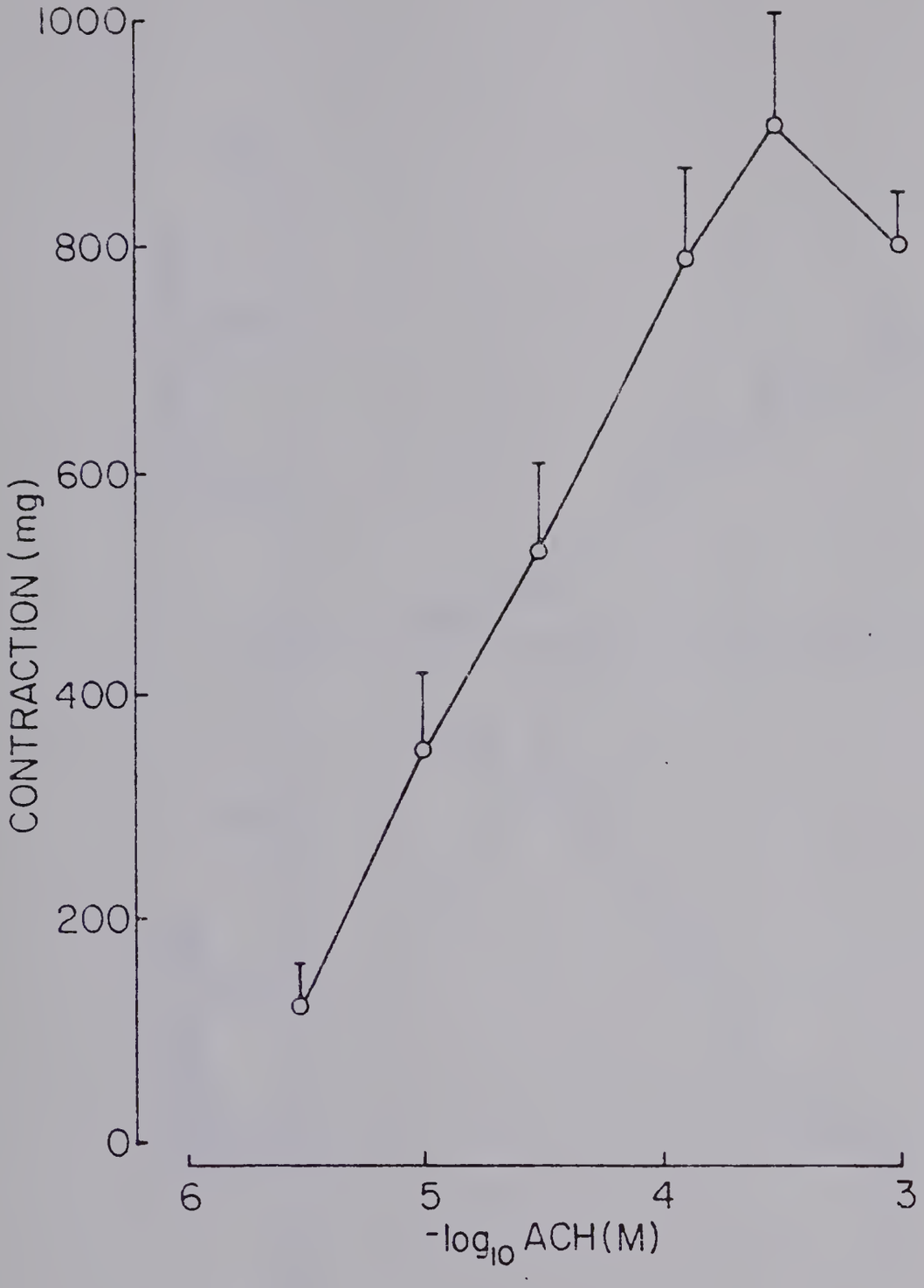

FIGURE 3

Effects of different concentrations of acetylcholine (ACH) on the tone of guinea pig intact gallbladder preparations. Five gallbladders were used, vertical lines indicate s.e. mean.

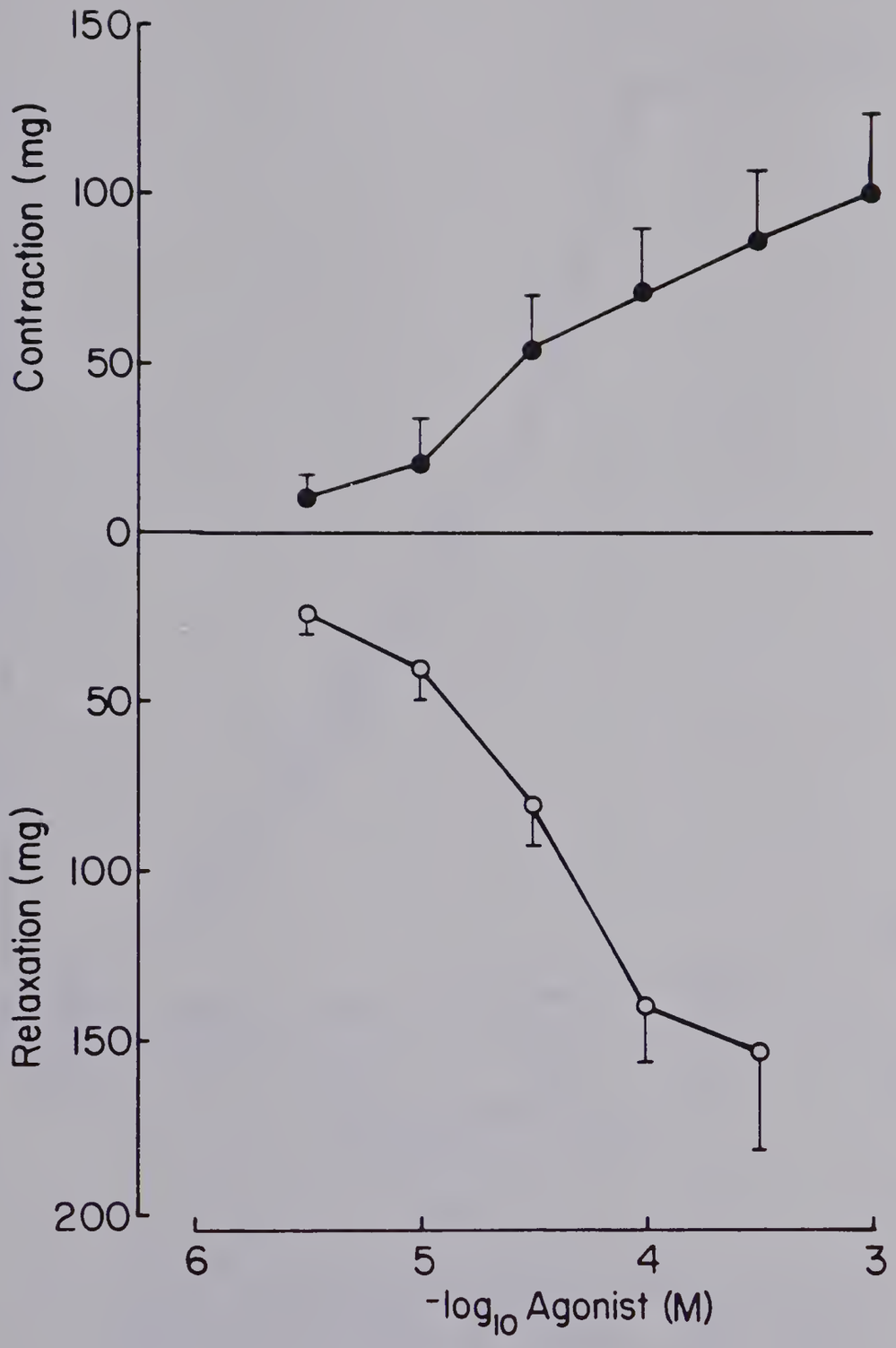

FIGURE 4

Concentration-response (mg) curves for the relaxations produced by adenosine (o) and contractions produced by ATP (•) in guinea pig intact gallbladders. Mean values (n=5) are given, vertical lines show s.e. mean.

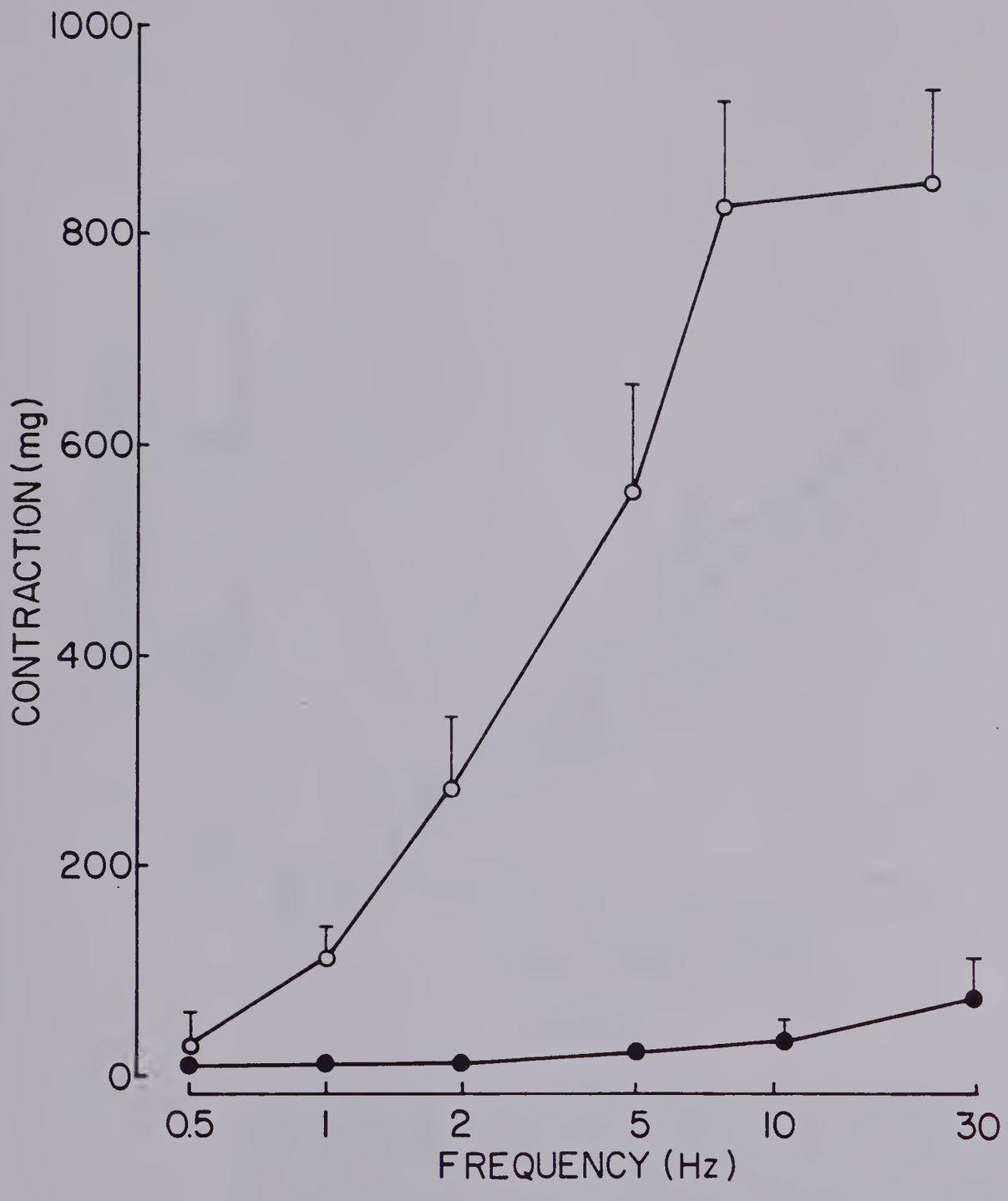

FIGURE 5

Frequency (log scale) response (mg) curve of the guinea pig intact gallbladder (o), and in the presence of atropine (•, $3X10^{-6}M$). Mean values (n=5) are given, vertical lines indicate s.e. mean.

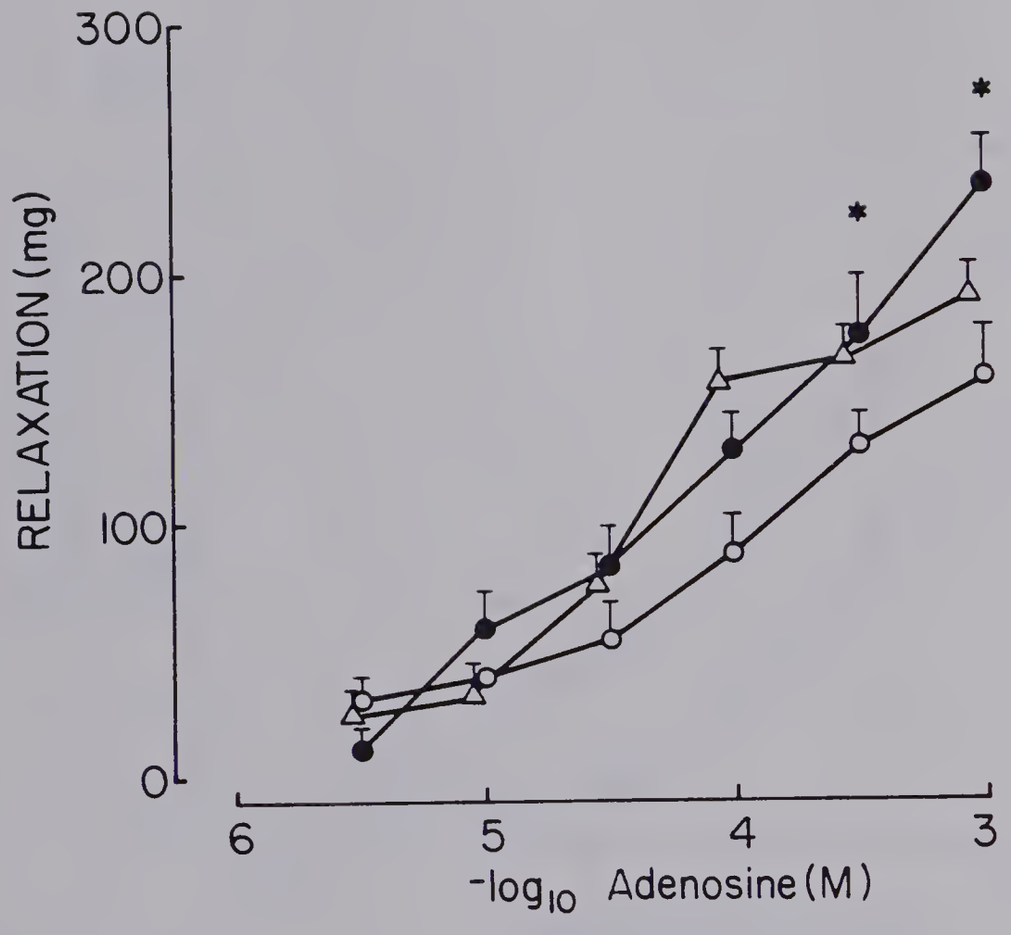

FIGURE 6

Effects of different concentrations of adenosine on the tone (mg) of guinea pig gallbladder strips. Control (O), time control (●) and following elevation of the tone with CCK (△, 0.06 u/ml). Mean values (n=12) are given, vertical lines denote s.e. mean. Asterisks denote significant ($P < 0.05$) differences from the corresponding time control.

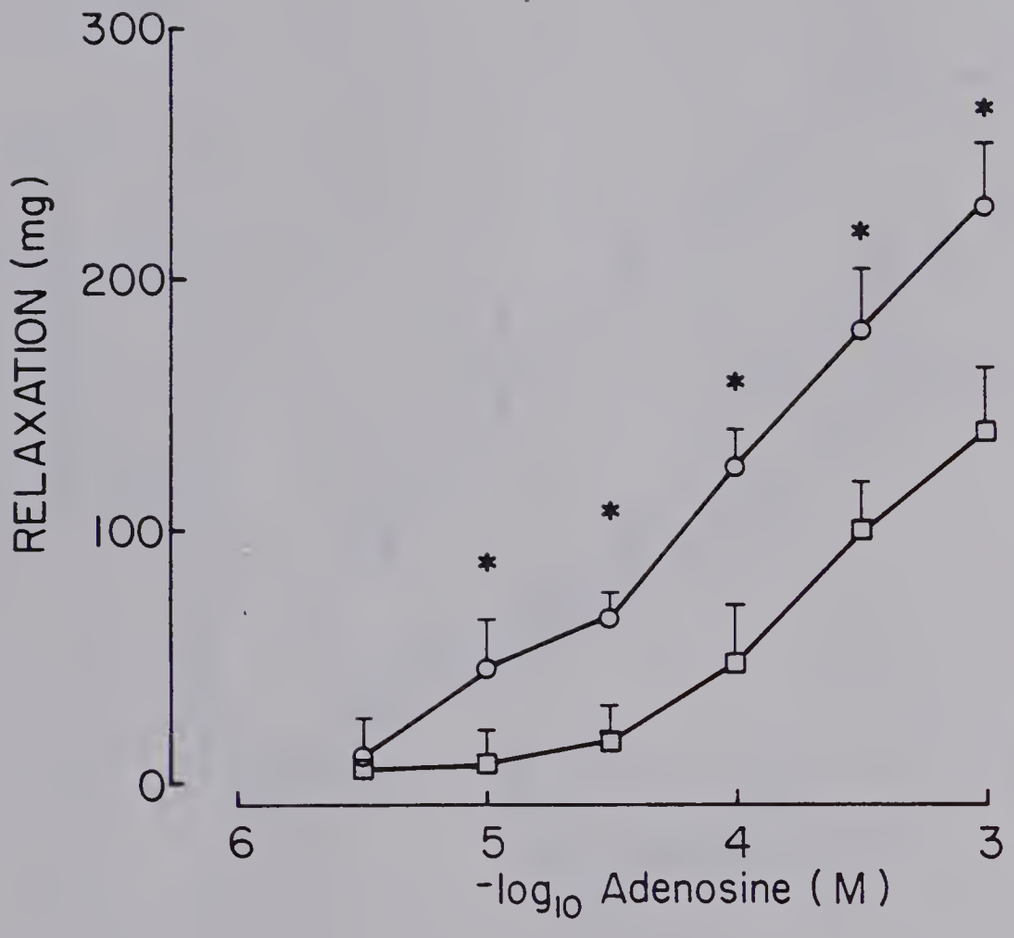

FIGURE 7

Effect of different concentrations of adenosine (o) alone, and following pretreatment with aminophylline (□, 10^{-5}M) on the tone (mg) of guinea pig gallbladder strips. Mean values (n=6) are shown, vertical lines indicate s.e. mean. Asterisks denote significant ($P < 0.05$) differences from the corresponding time control.

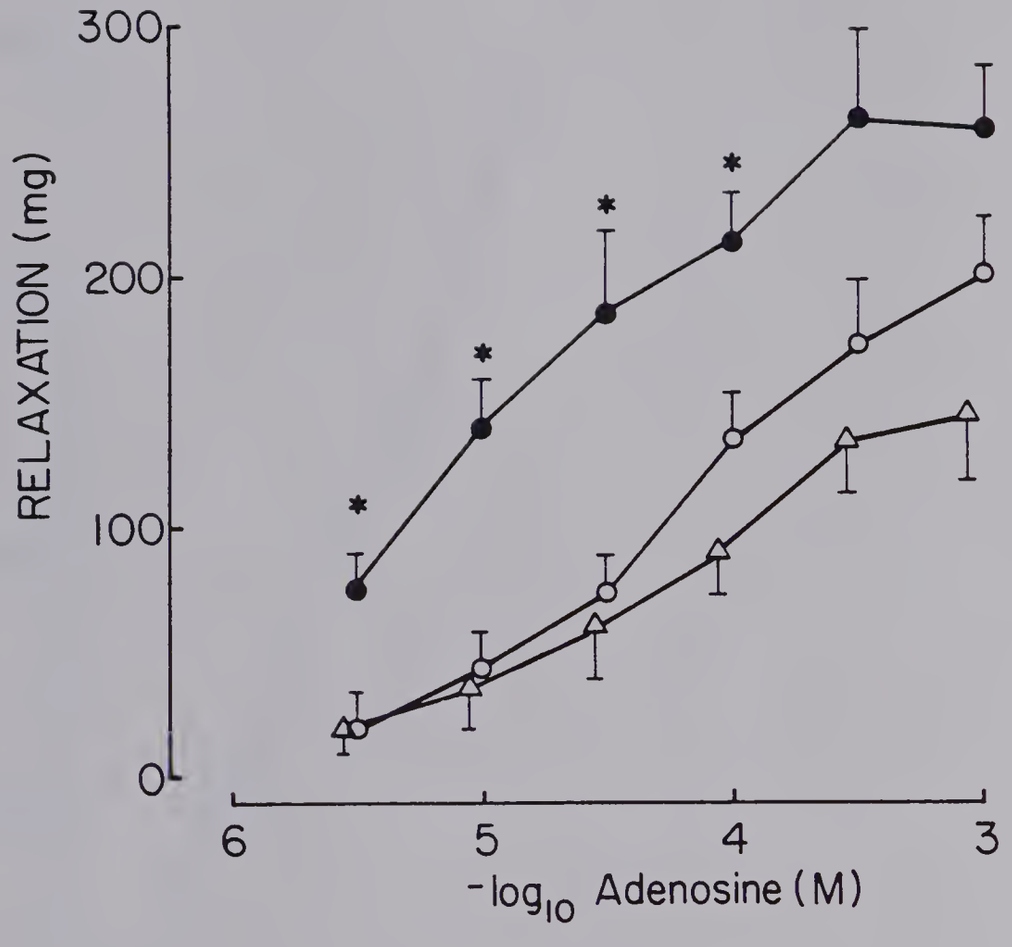

FIGURE 8

Effects of different concentrations of adenosine (o) alone, following pretreatment with HNBTG ($\bullet 10^{-6}$M) and HNBTG + aminophylline ($\Delta 10^{-5}$M) on the tone (mg) of guinea pig gallbladder strips. Mean values (n=6) are given, vertical lines indicate s.e. mean. Asterisks denote significant ($P < 0.05$) differences from the corresponding time controls (unpaired values).

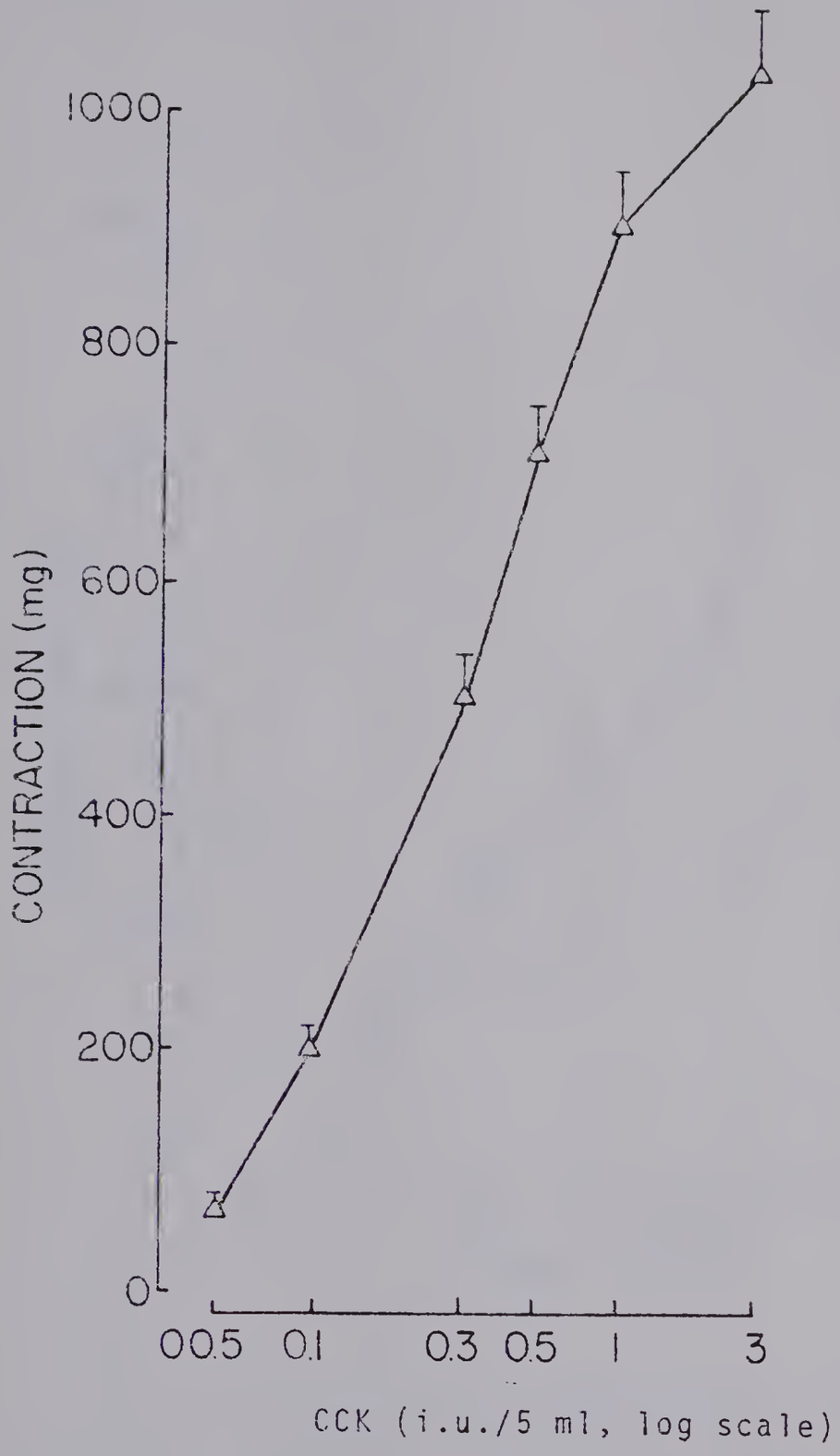

FIGURE 9

Effects of different concentrations of CCK (Δ) on the tone of guinea pig gallbladder strips. Mean values (n=4) are given, vertical lines show s.e. mean.

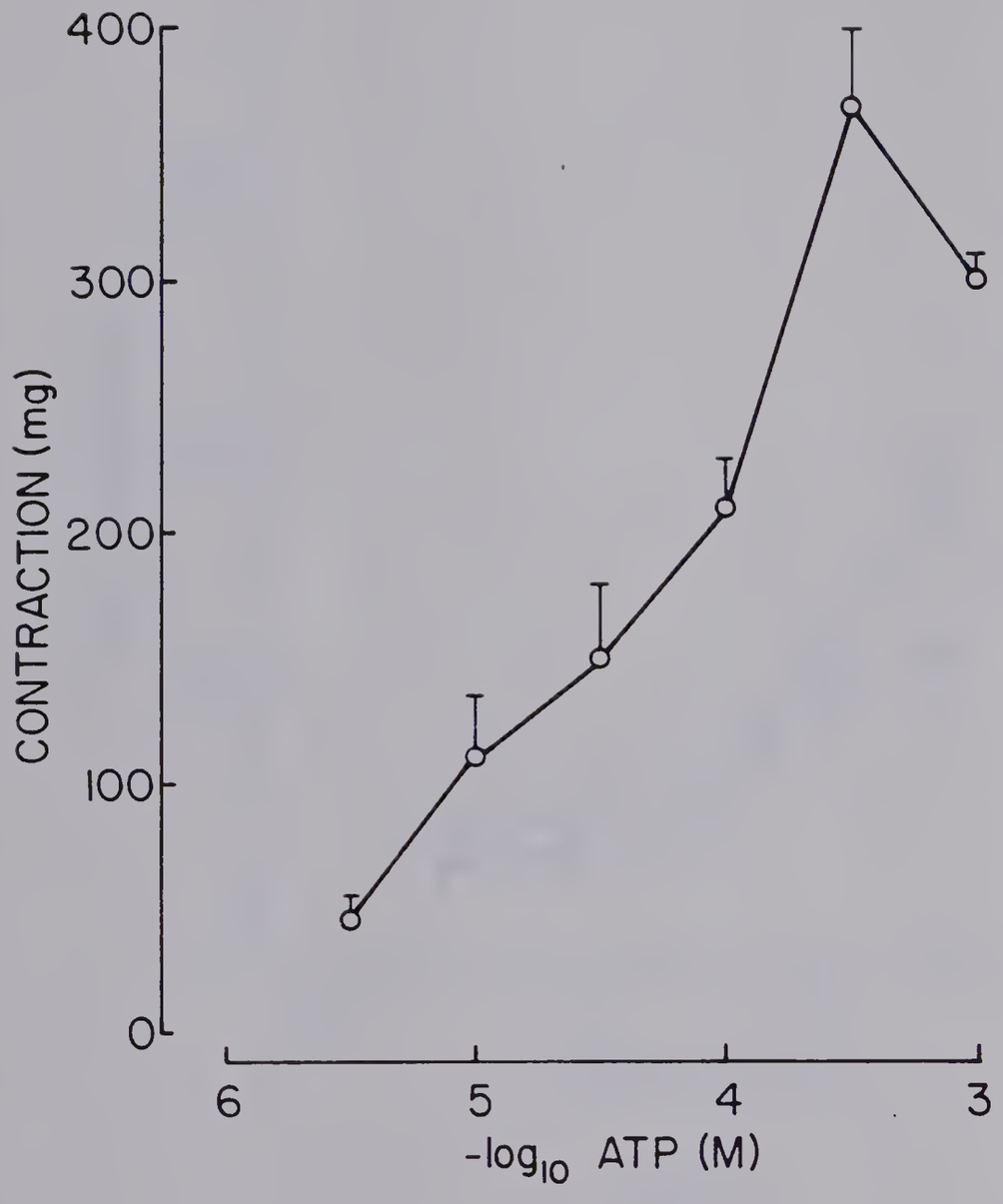

FIGURE 10

Effects of different concentrations of ATP (o) on the tone (mg) of guinea pig gallbladder strips. Six strips from six animals were used; vertical lines show s.e. mean.

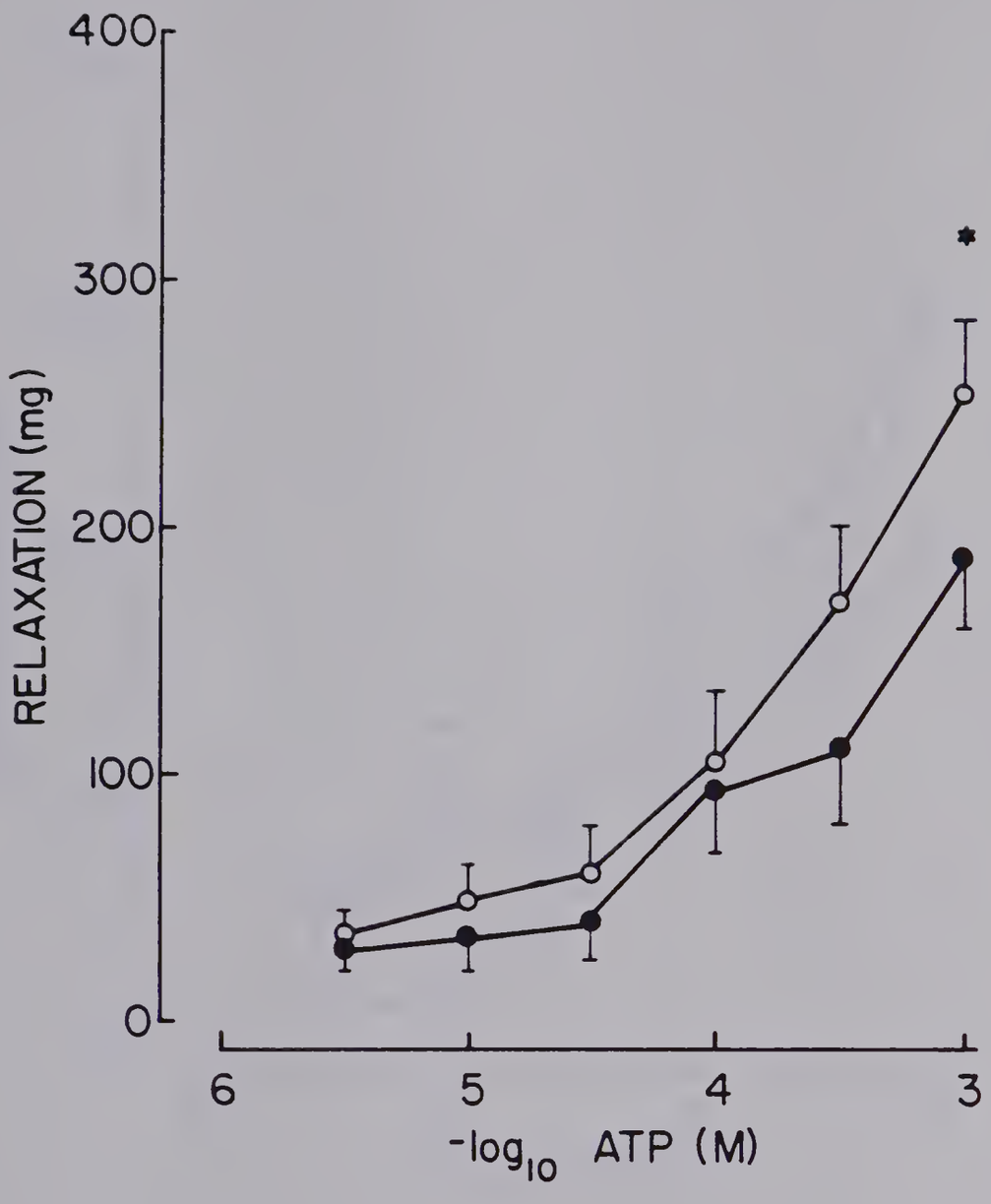

FIGURE 11

Effects of different concentrations of ATP (o), (tissue pretreated with indomethacin $3X10^{-6}$ and tone raised with CCK 0.06 u/m), and in the presence of aminophylline (•10^{-5}M). Mean values (n=6) are given, vertical lines show s.e. mean. Asterisk denotes significant ($P<0.05$) difference between groups.

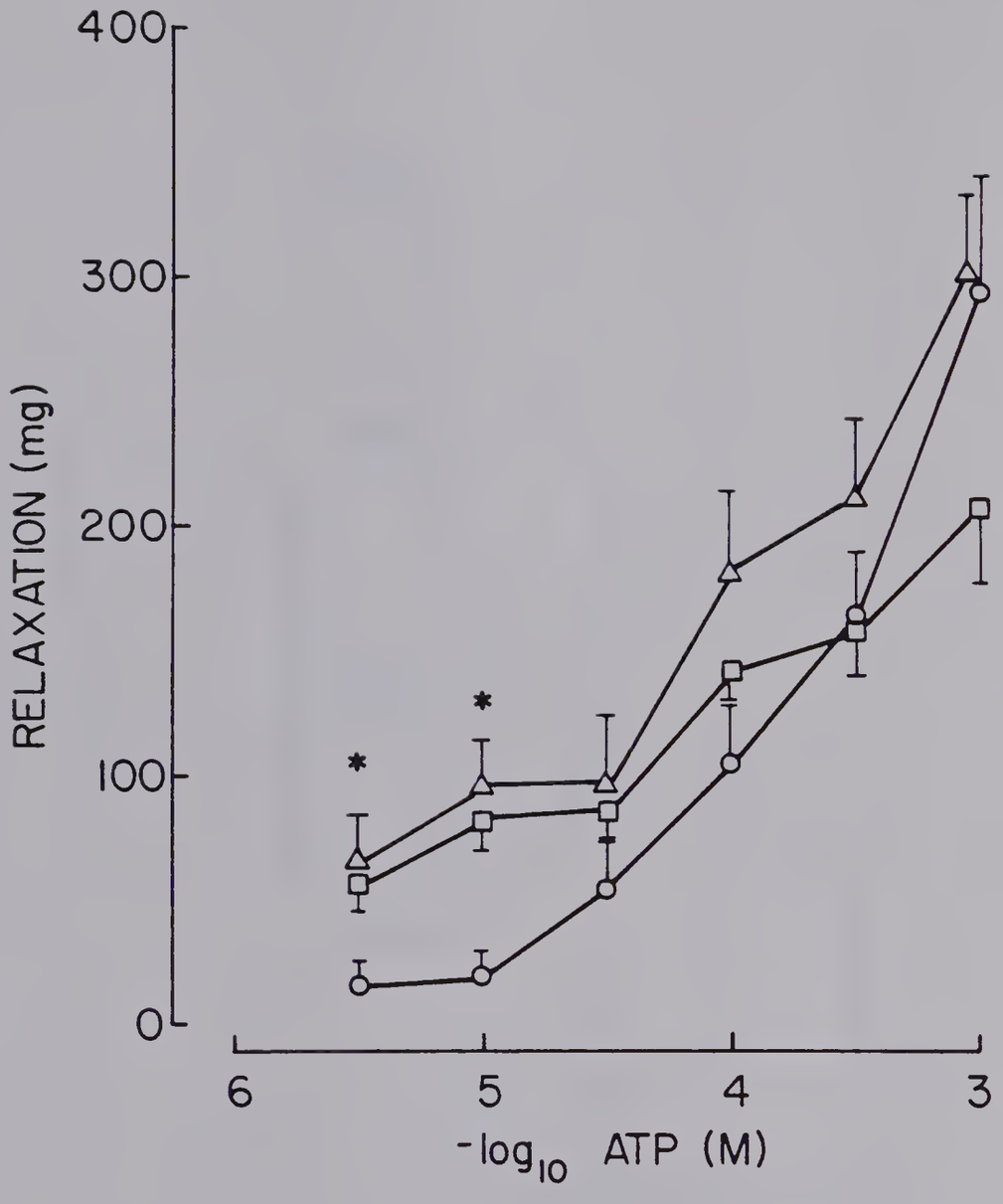

FIGURE 12

Relaxant responses (mg) of different concentrations of ATP (o), pretreatment with HNBTG (Δ, 10^{-6}M); HNBTG (10^{-6}M) + aminophylline (□, 10^{-5}M). All tissues were pretreated with indomethacin ($3X10^{-6}$M) and tone raised with CCK (0.06 u/ml). Mean values (n=6) are given, vertical lines show s.e. mean Asterisks denote significant (P 0.05) differences between groups (unpaired values).

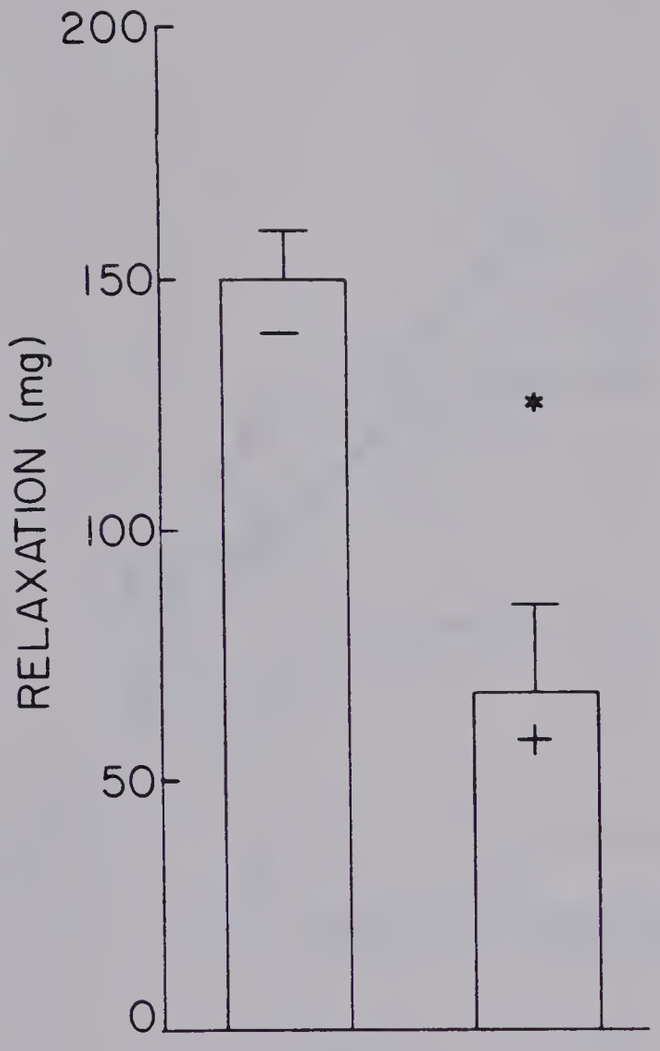

FIGURE 13

Histogram depicting relaxant response (mg) to 10^{-4} ATP (- alone and after pretreatment of the tissues with 8 u/ml of adenosine deaminase (+), which was sufficient to abolish the responses to adenosine. Mean values (n=6) are given; vertical lines show s.e. mean. Asterisk denotes significance ($P<0.05$) difference between groups.

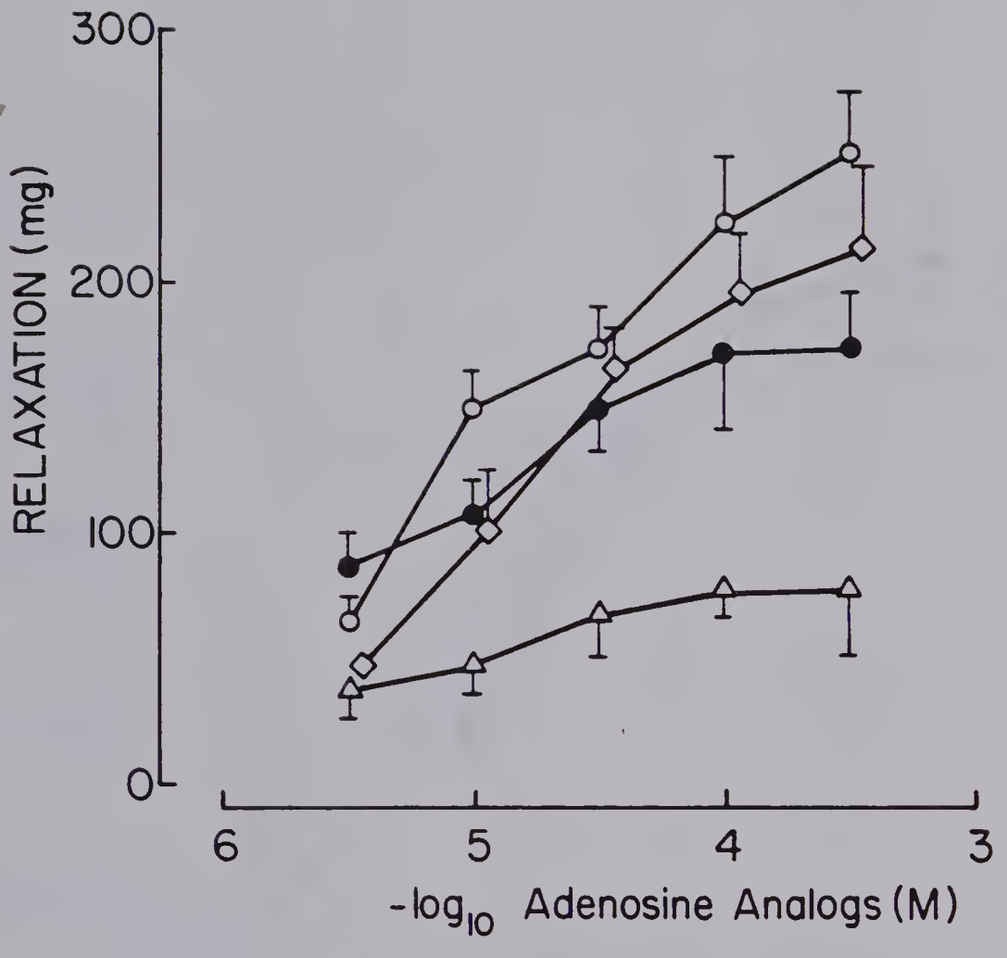

FIGURE 14

Effect of different concentrations of 2-chloroadenosine (O), NECA (◇), adenosine + HNBTG (●) and PIA (△) on the tone (mg) of guinea pig gallbladder strips. Mean values (n=4) are given; vertical lines show s.e. mean.

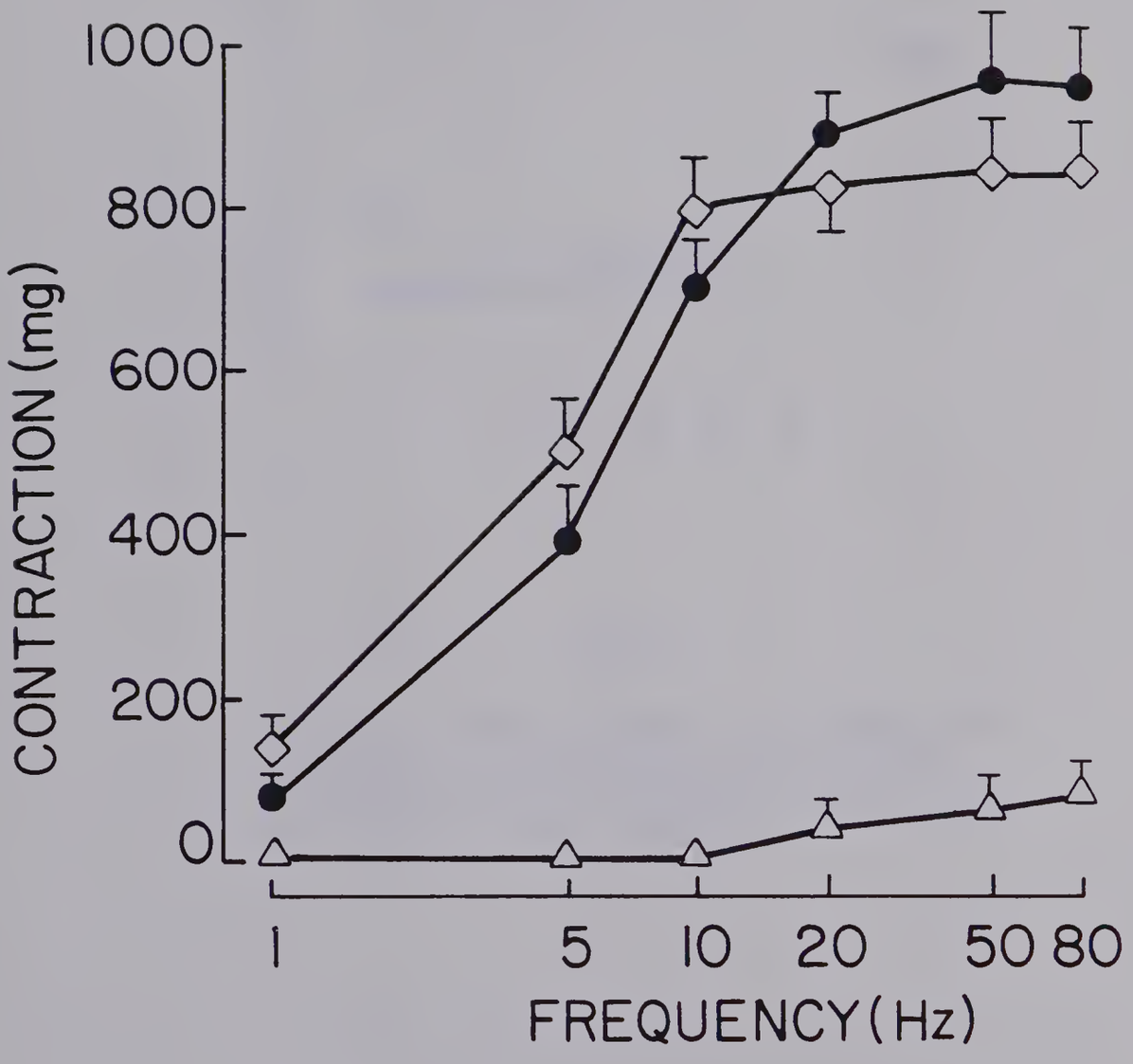

FIGURE 15

Frequency—Response (mg) curves of guinea-pig gallbladder strips. Control (●), time control (◇), and following pretreatment with TTX (Δ, 10^{-6}M). Mean values (n=10) are given; vertical lines denote s.e. mean.

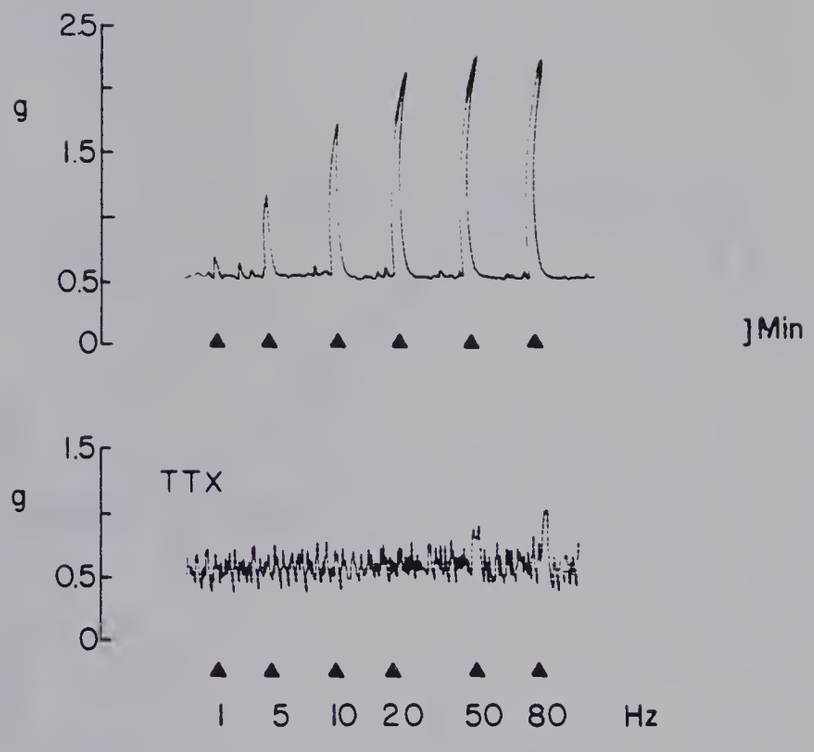

FIGURE 16

Typical frequency-dependent responses (mg) of guinea-pig gallbladder strips (upper tracing), and following pretreatment with TTX (10^{-6}M; lower tracing).

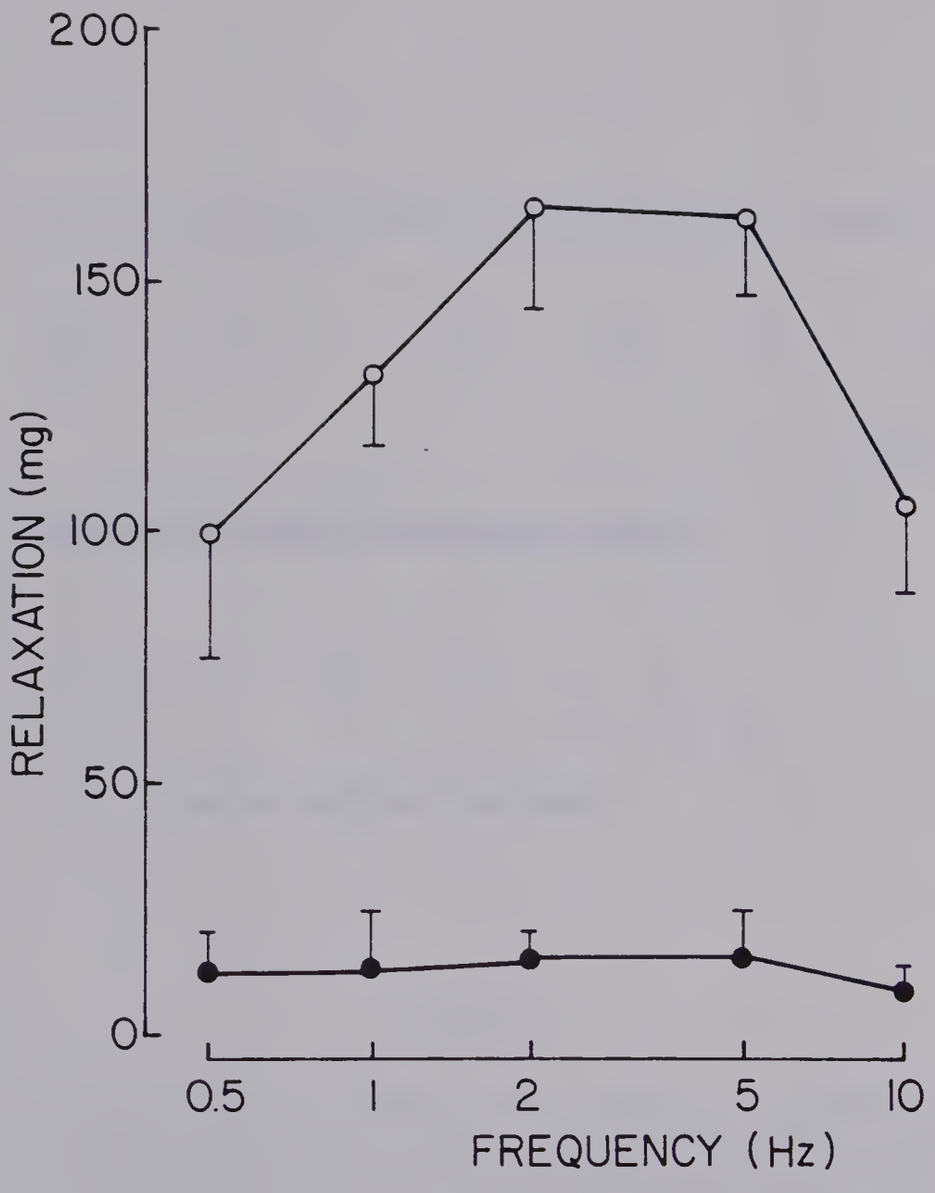

FIGURE 17

Frequency–response (mg) curves of guinea-pig gallbladder strips in the presence of atropine ($3X10^{-6}M$) and guanethidine ($2X10^{-5}M$) (o), and following pretreatment with TTX (●, $10^{-6}M$). Mean values (n=12) are given; vertical lines show s.e. mean.

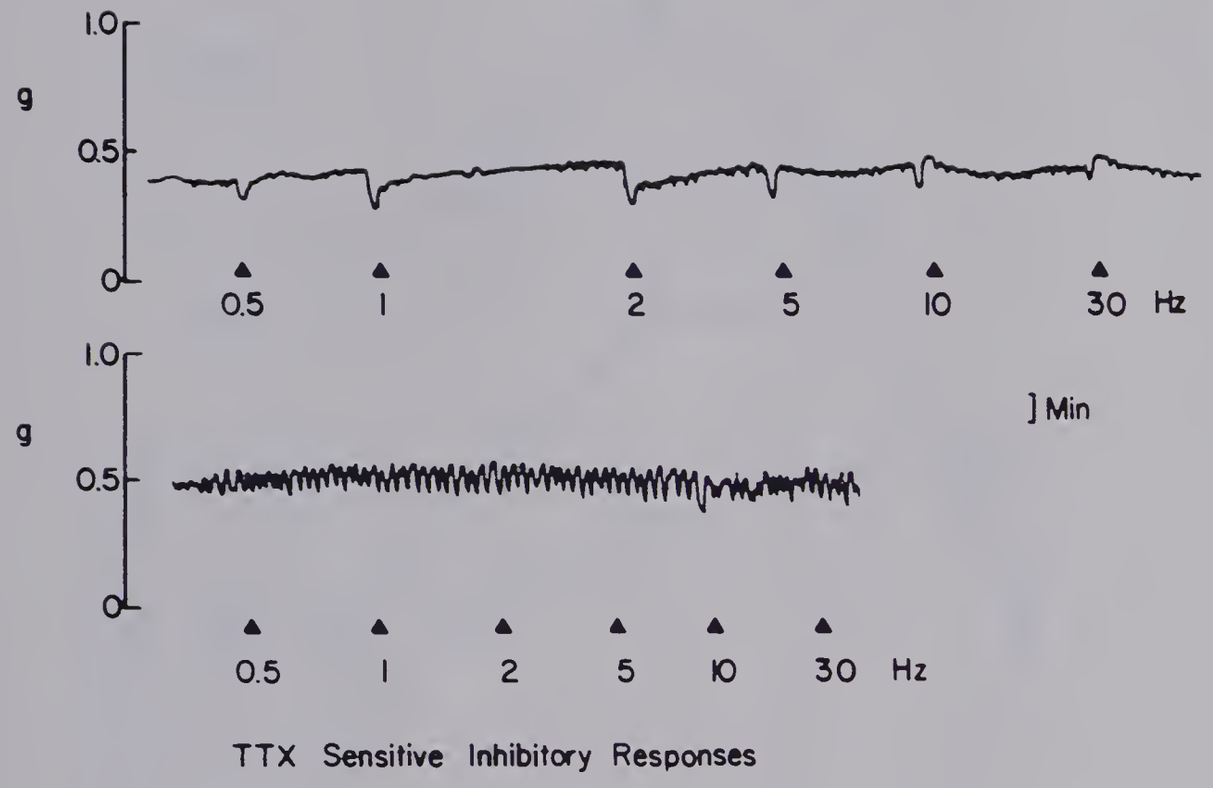

FIGURE 18

Typical frequency–response (mg) pattern of guinea-pig gallbladder strips following pretreatment with atropine ($3X10^{-6}M$) and guanethidine ($2X10^{-5}M$; upper tracing); relaxant responses abolished by TTX ($10^{-6}M$; lower tracing).

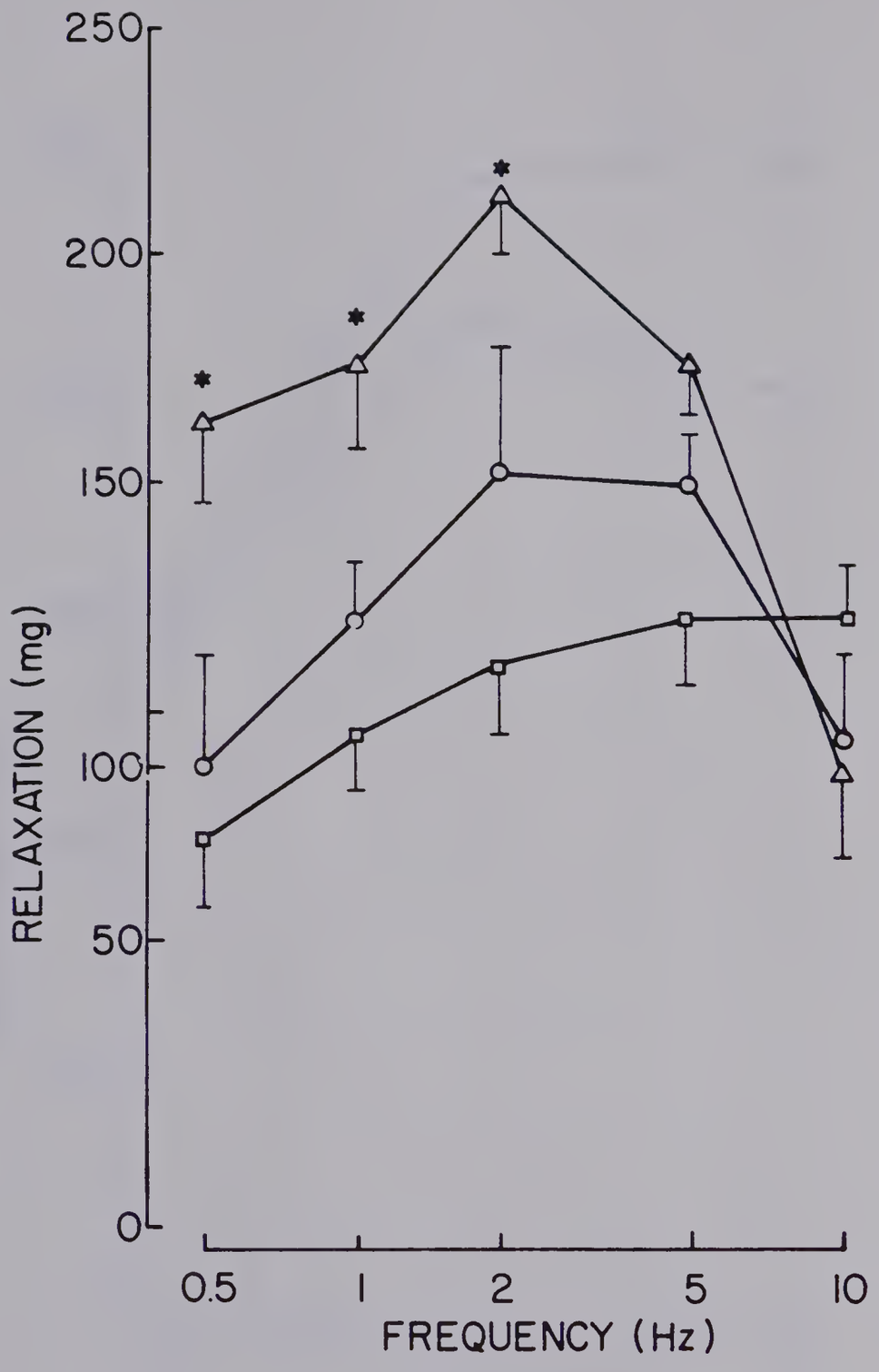

FIGURE 19

Frequency-response (mg) curves of guinea-pig gallbladder strips following pretreatment with atropine ($3X10^{-6}M$) and guanethidine ($2X10^{-5}M$) (o), in the presence of HNBTG (Δ, $10^{-6}M$) and HNBTG ($10^{-6}M$) + aminophylline (□, $10^{-5}M$). Mean values (n=6) are given; vertical lines indicate s.e. mean. Asterisks denote significant ($P<0.05$) differences between groups.

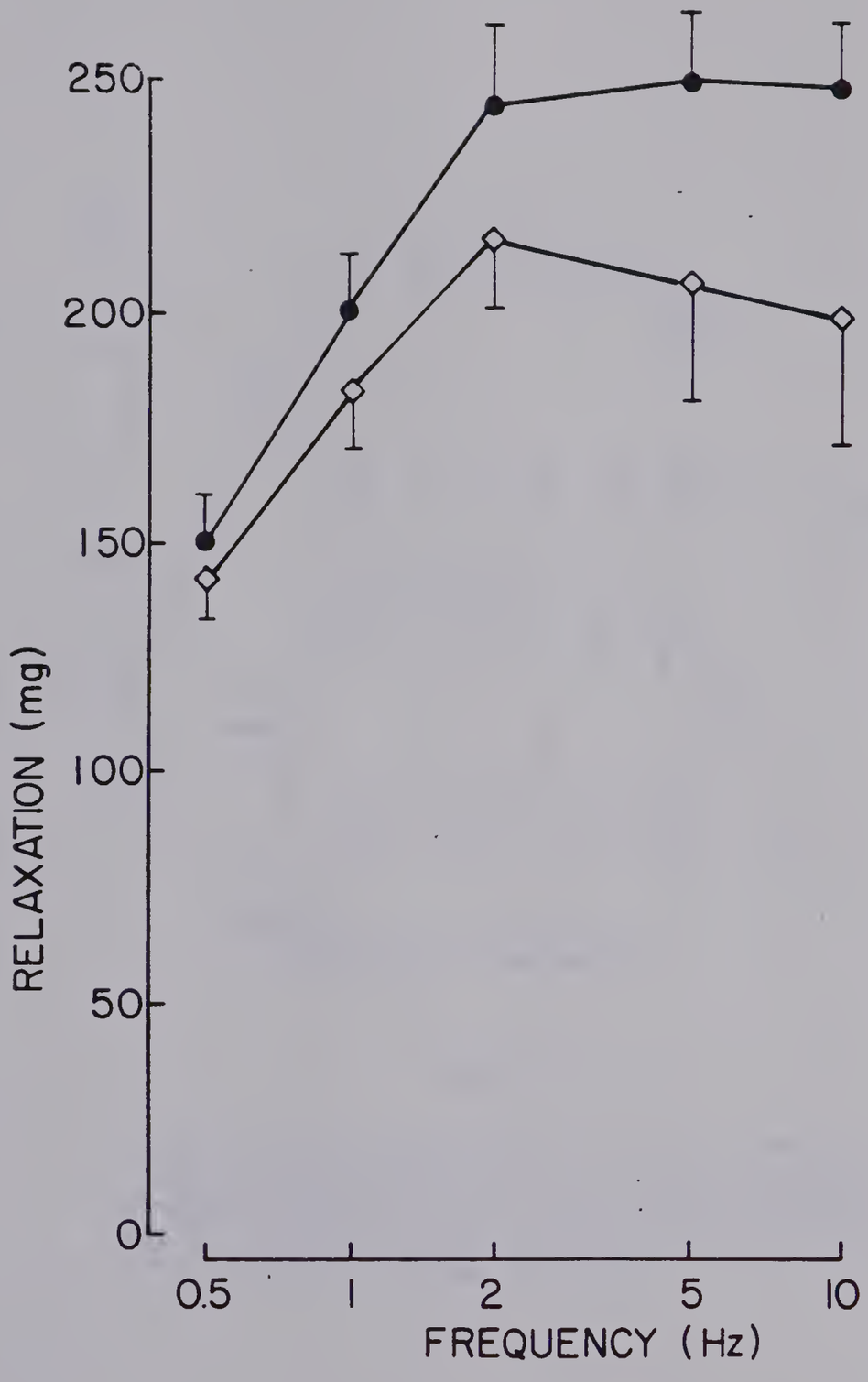

FIGURE 20

Frequency-response (mg) curves of guinea-pig gallbladder strips in the presence of atropine ($3X10^{-6}M$), guanethidine ($2X10^{-5}M$), and CCK (●, 0.06 U/ml), and responses following pretreatment with TTX (◇, $10^{-6}M$). Mean values (n=12) are given; vertical lines show s.e. mean.

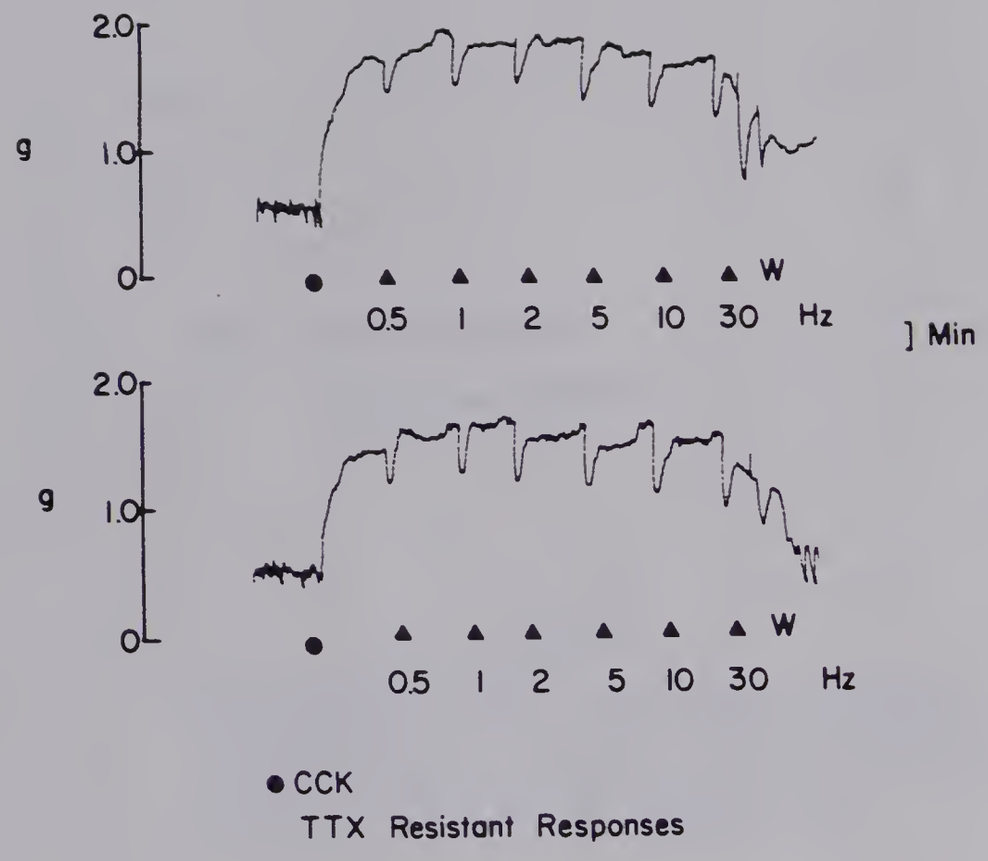

FIGURE 21

Typical relaxant responses on field stimulation of guinea-pig gallbladder strips in the presence of atropine ($3X10^{-6}M$) and guanethidine ($2X10^{-5}M$) and CCK (0.06 U/ml) (upper tracing); following pretreatment with TTX ($10^{-6}M$), lower tracing .

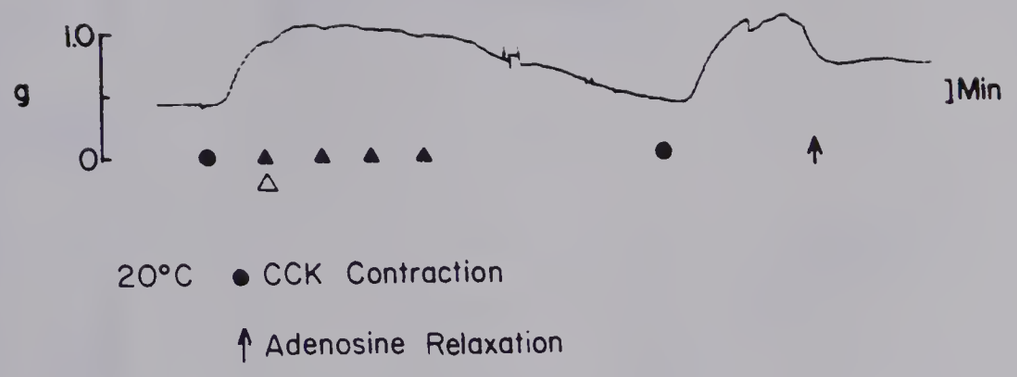

FIGURE 22

Experiment at 20°C. No relaxant responses demonstrated on electrical field stimulation. Tissue contracts in response to CCK (●) and relaxes in response to adenosine (↑).

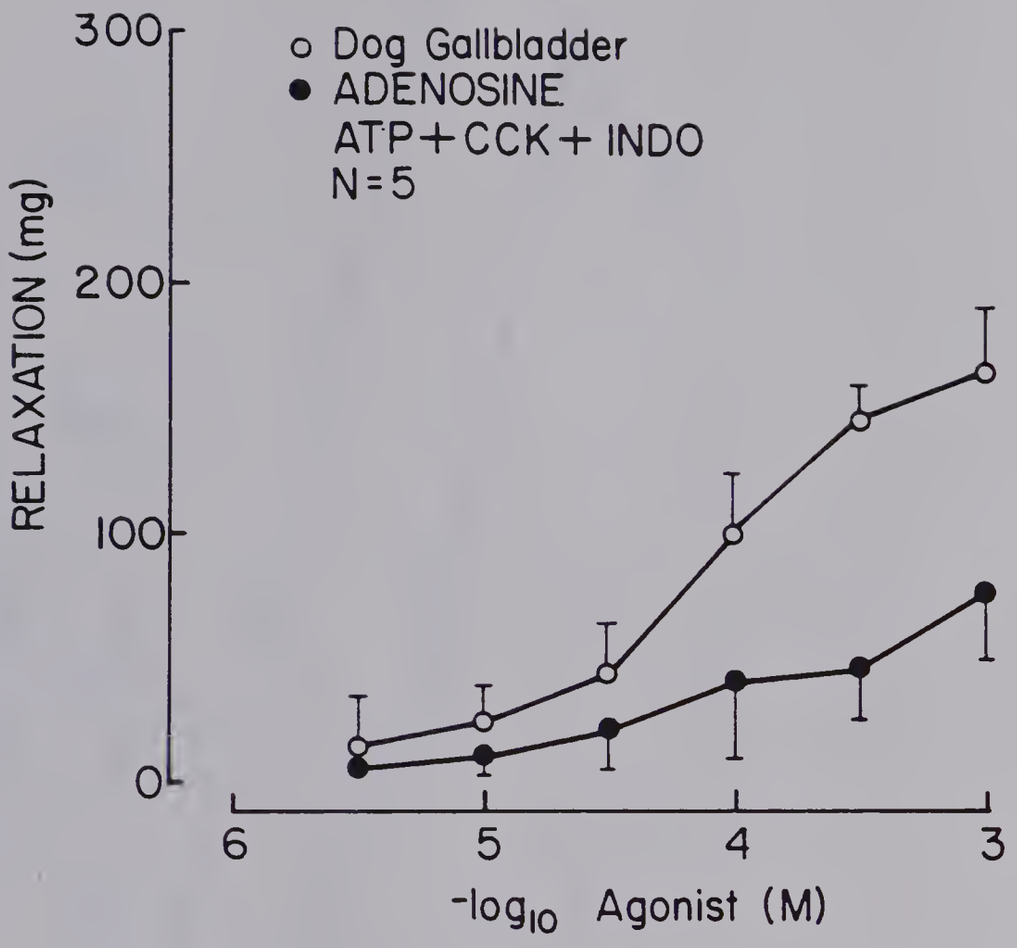

FIGURE 23

Effects of different concentrations of adenosine (o) on the tone (mg) of dog gallbladder strips. ATP (●)-mediated relaxations in the presence of indomethacin ($3X10^{-6}M$) and tone raised with CCK (0.06 U/ml). Mean values (n=5) are given; vertical lines show s.e. mean.

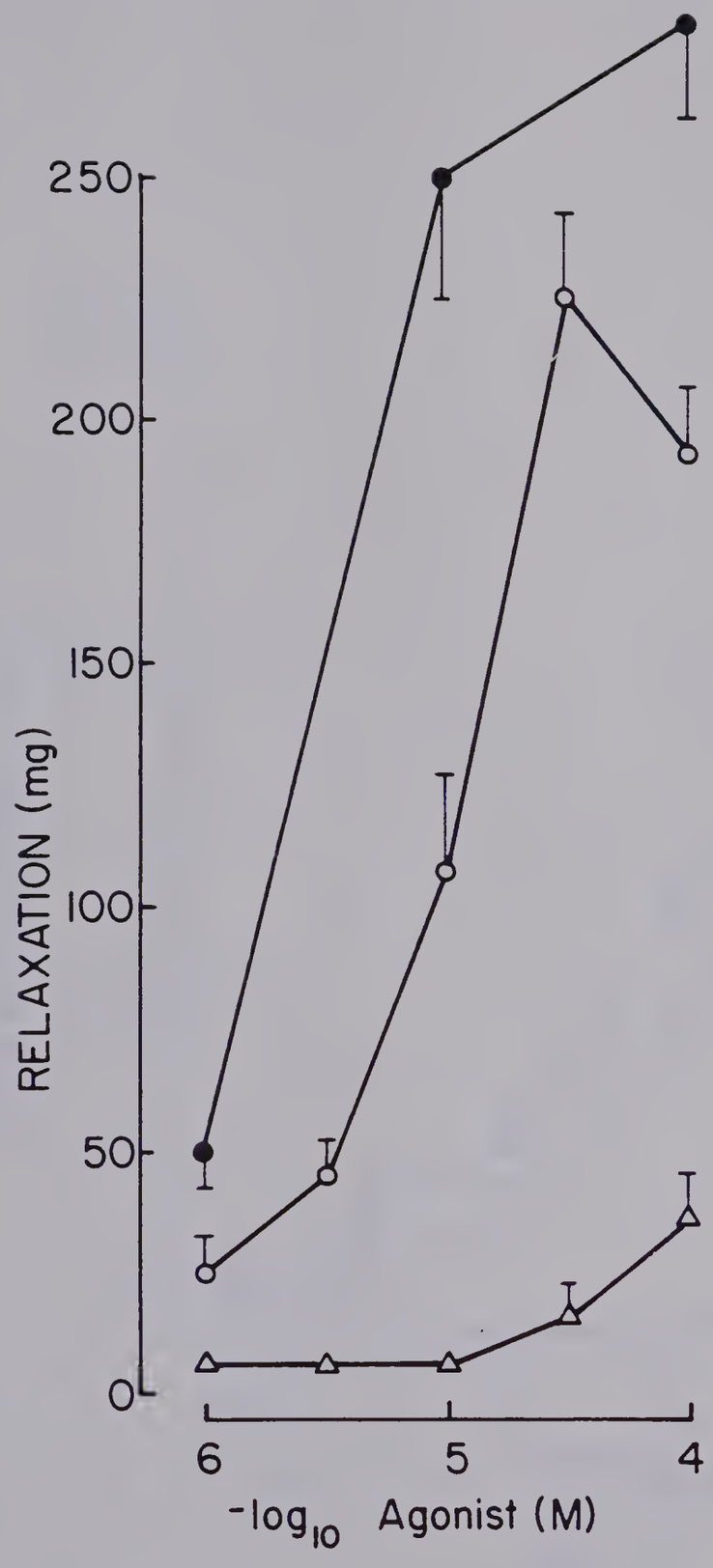

FIGURE 24

Effects of different concentrations of isoproterenol (o, INA), time-dependent changes (•), following pretreatment with propranolol (△, $2X10^{-5}$), on the tone (mg) of dog gallbladder strips. Mean values (n=5) are given; vertical lines indicate s.e. mean.

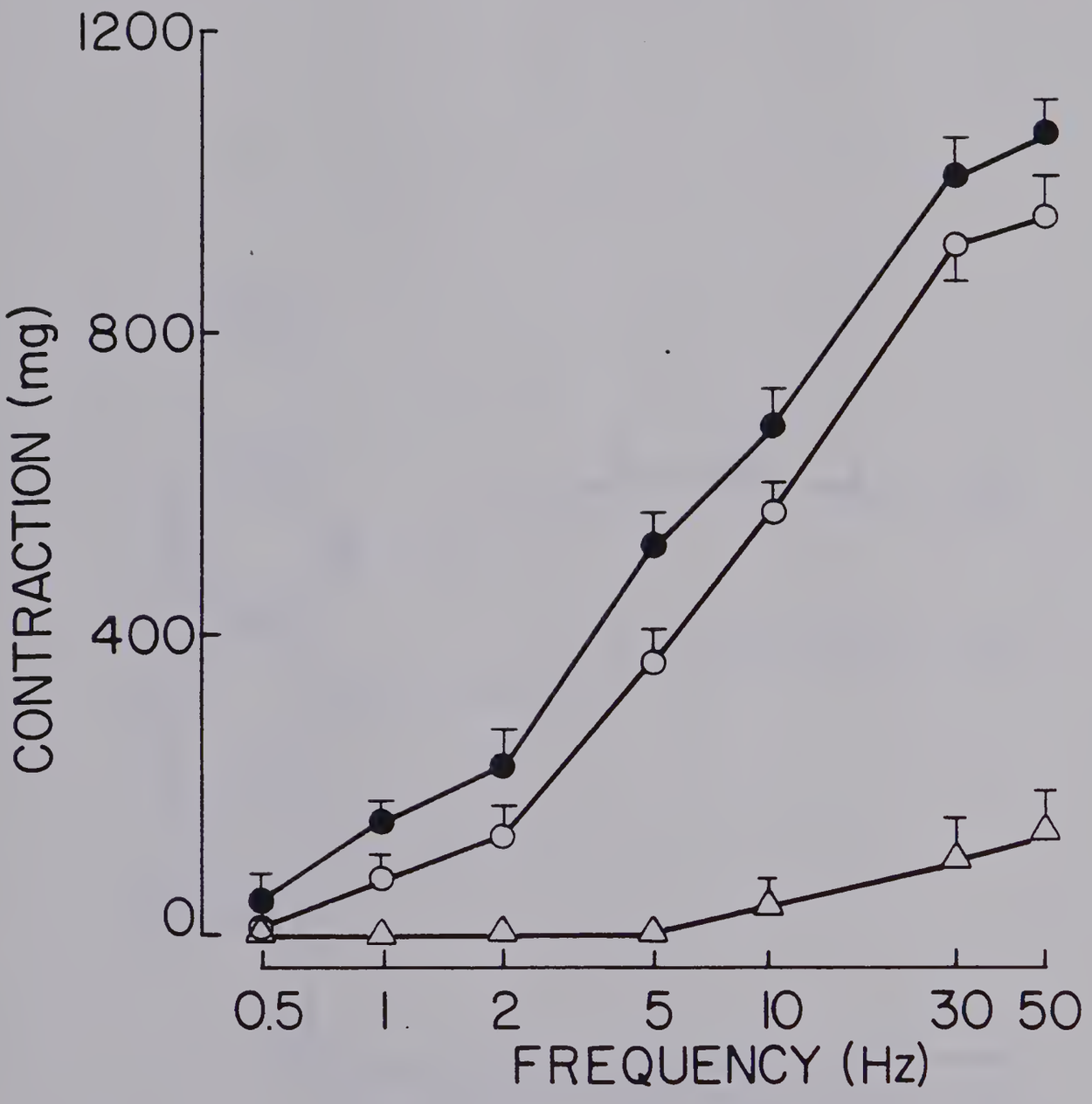

FIGURE 25

Frequency–response (mg) curves of dog gallbladder strips with a longitudinally directed electrical field (●); transverse field stimulation (o) alone, and in the presence of TTX (Δ, 10^{-6}M). Mean values (n=5) are given; vertical lines show s.e. mean.

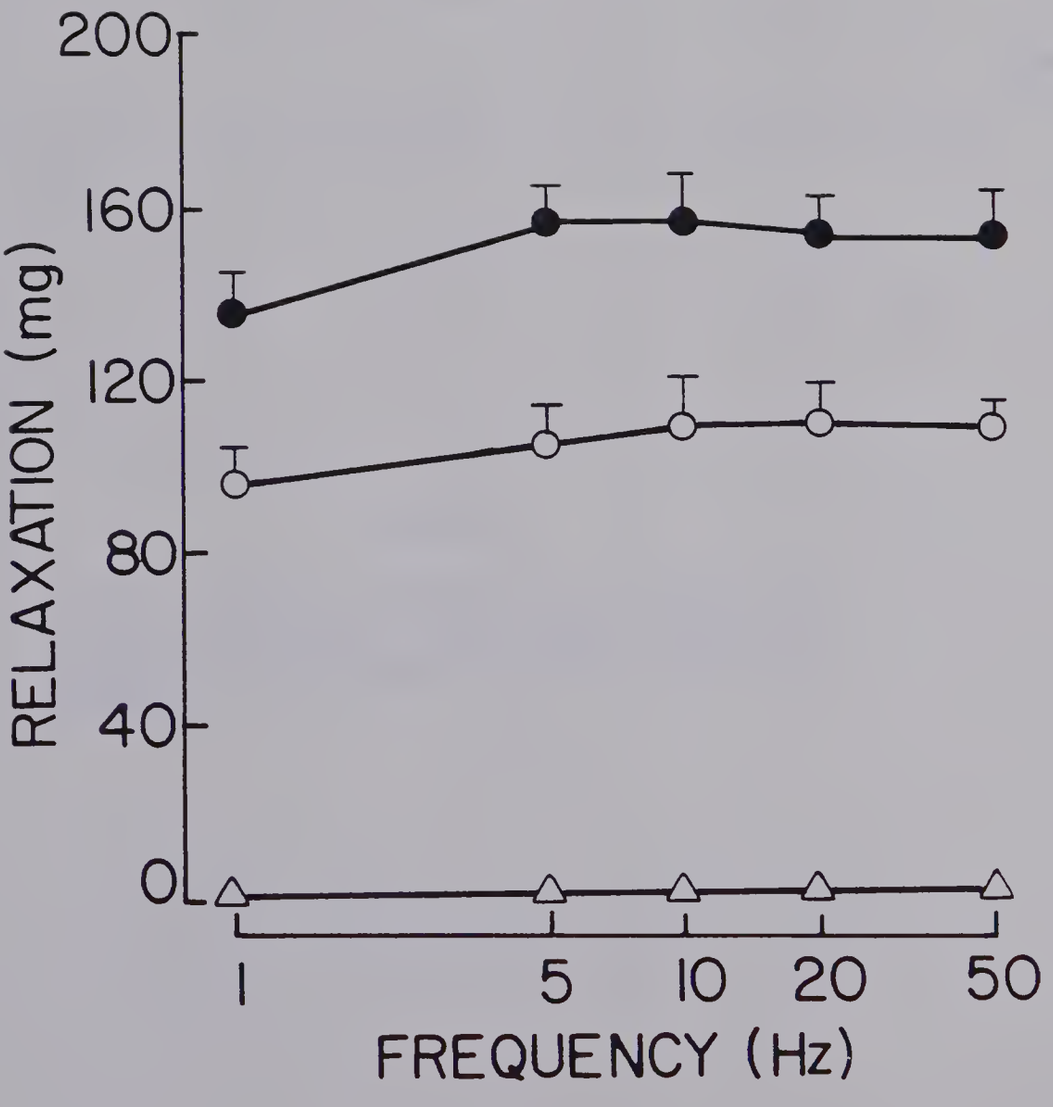

FIGURE 26

Frequency–response (mg) curves in the presence of atropine ($3X10^{-6}M$) and guanethidine ($2X10^{-5}M$) to longitudinal field stimulation (●); transverse field stimulation alone (o), and in the presence of TTX (△, $10^{-6}M$). Mean values (n=6) are given; vertical lines show s.e. mean.

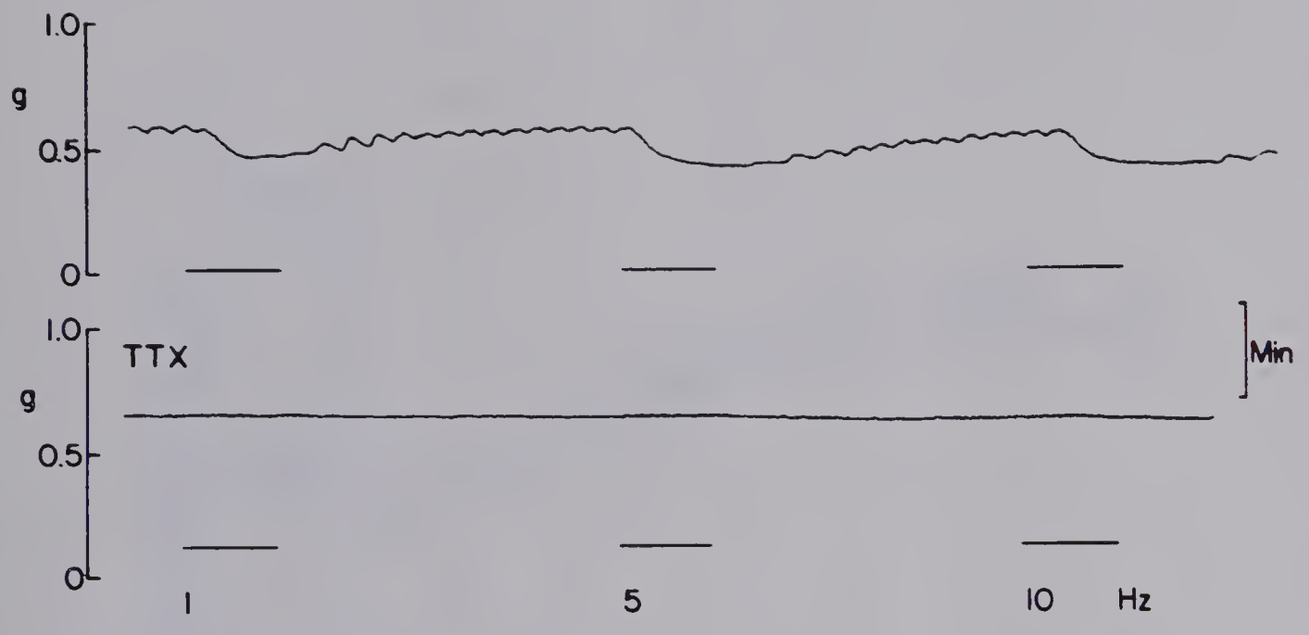

FIGURE 27

Typical relaxant responses to a transversely directed electrical field (upper tracing); following pretreatment with TTX (10^{-6}M lower tracing).

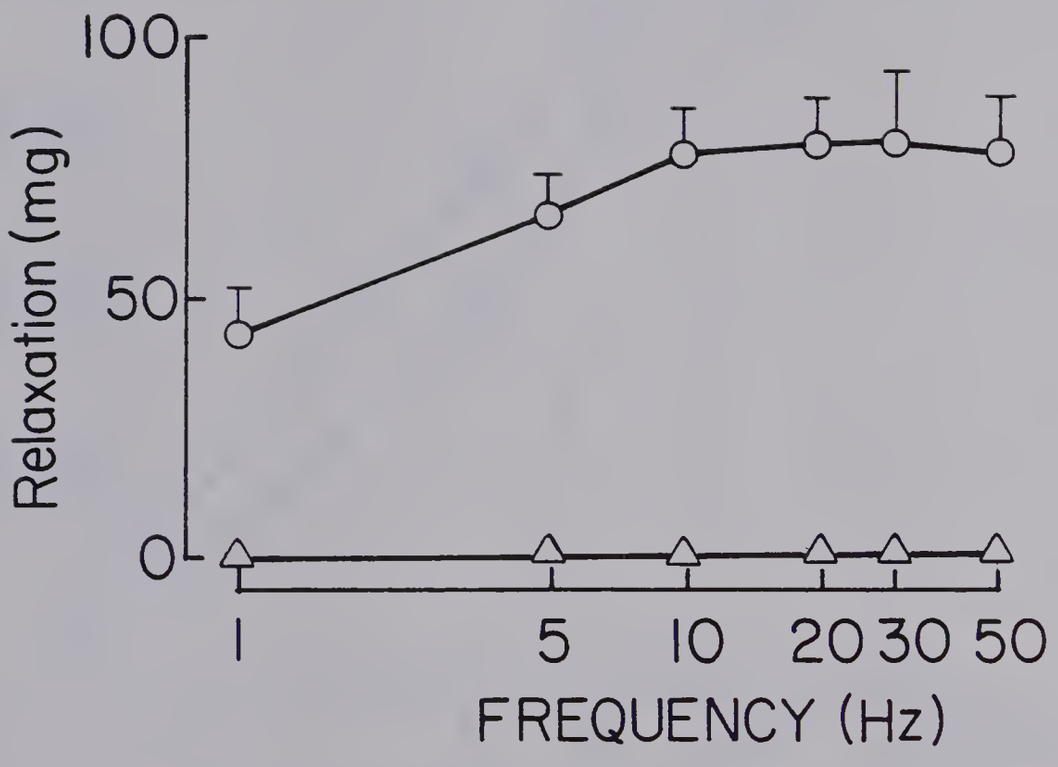

FIGURE 28

Frequency–response (mg) curve in the presence of atropine ($3X10^{-6}M$) and guanethidine ($2X10^{-5}M$) of dog cystic-duct strips (o) alone; following pretreatment with TTX (Δ, $10^{-6}M$): transverse field stimulation. Mean values (n=5) are given; vertical lines show s.e. mean.

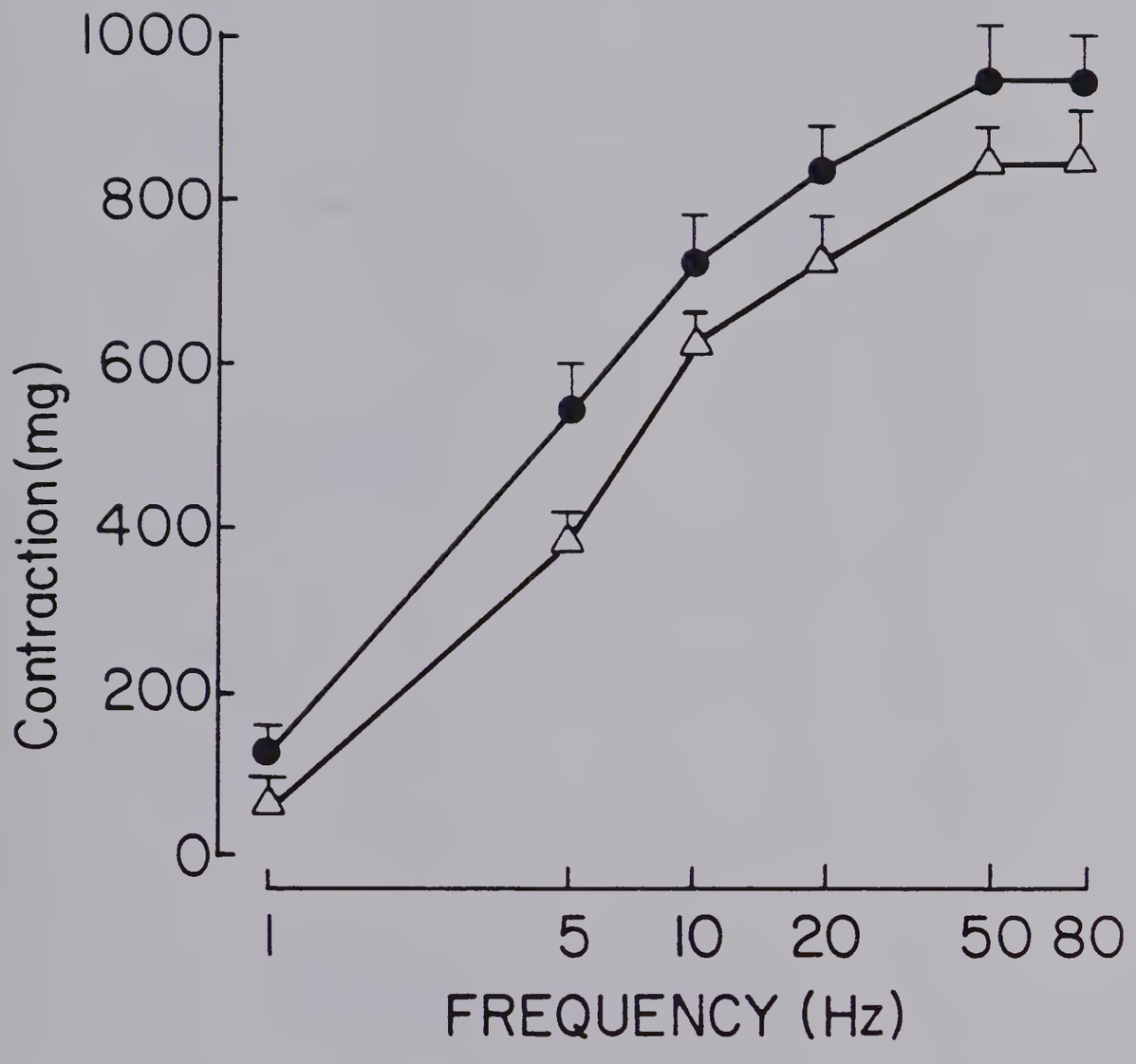

FIGURE 29

Frequency–responses (mg) curves of dog gallbladder strips with a longitudinally directed electrical field at a pulse duration of 1 ms (●), and 0.5 ms (△). Mean values are given (n=6); vertical lines show s.e. mean.

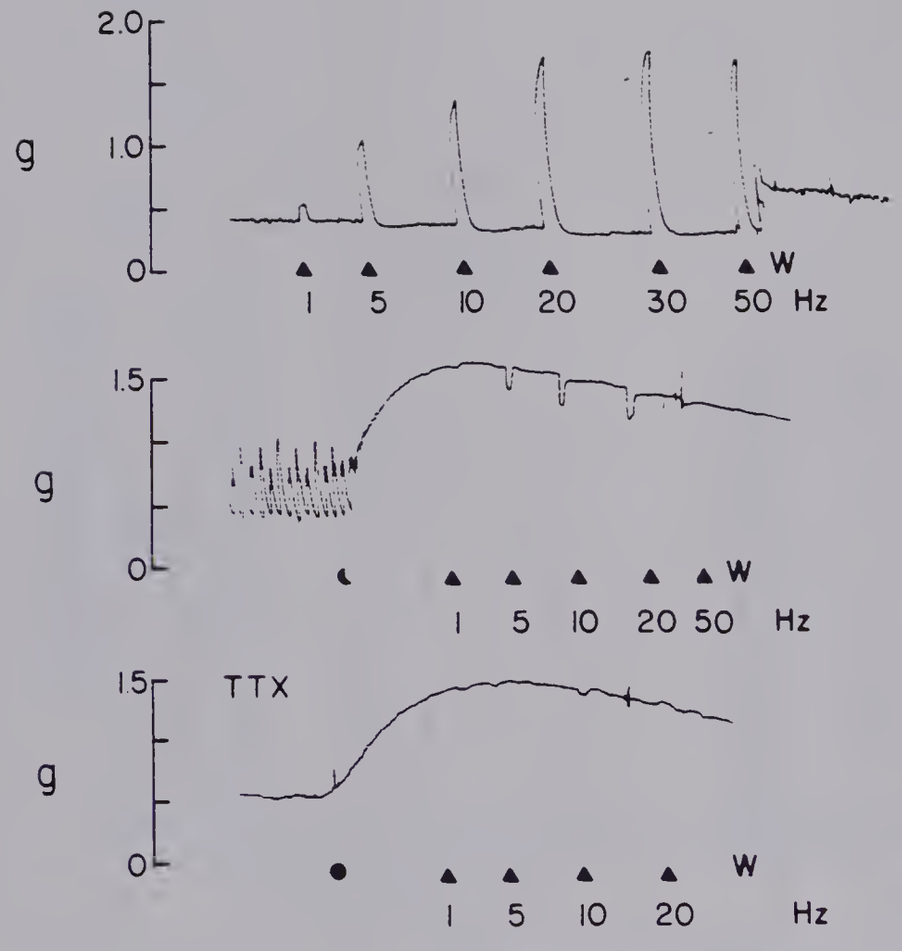

FIGURE 30

Typical frequency-response pattern of dog gallbladder strips to electrical field stimulation (upper tracing); relaxant responses in the presence of atropine ($3X10^{-6}M$) guanethidine ($2X10^{-5}M$) and CCK (●, 0.06 U/ml) at 0.5 ms (middle tracing); following pretreatment with TTX ($10^{-6}M$) (lower tracing).

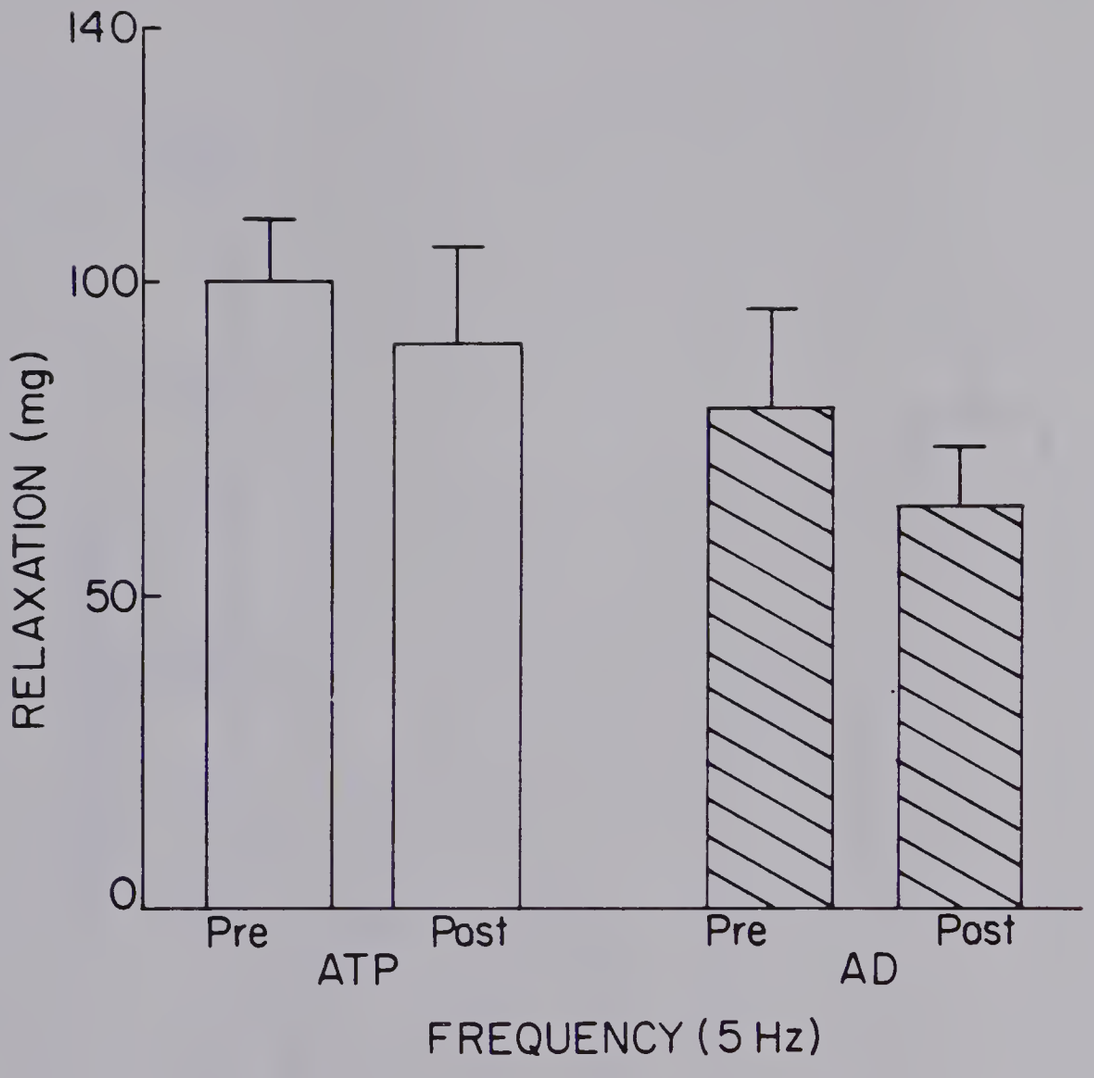

FIGURE 31

Histogram depicting relaxant-responses (mg) of dog gallbladder strips to NANCI stimulation at a frequency of 5 Hz. Open columns represent responses obtained before (Pre) and after (Post) autoinhibition to ATP. Hatched columns represent similar responses before (Pre) and after (Post) autoinhibition to adenosine. Mean values (n=6) are given; vertical lines denote s.e. mean.

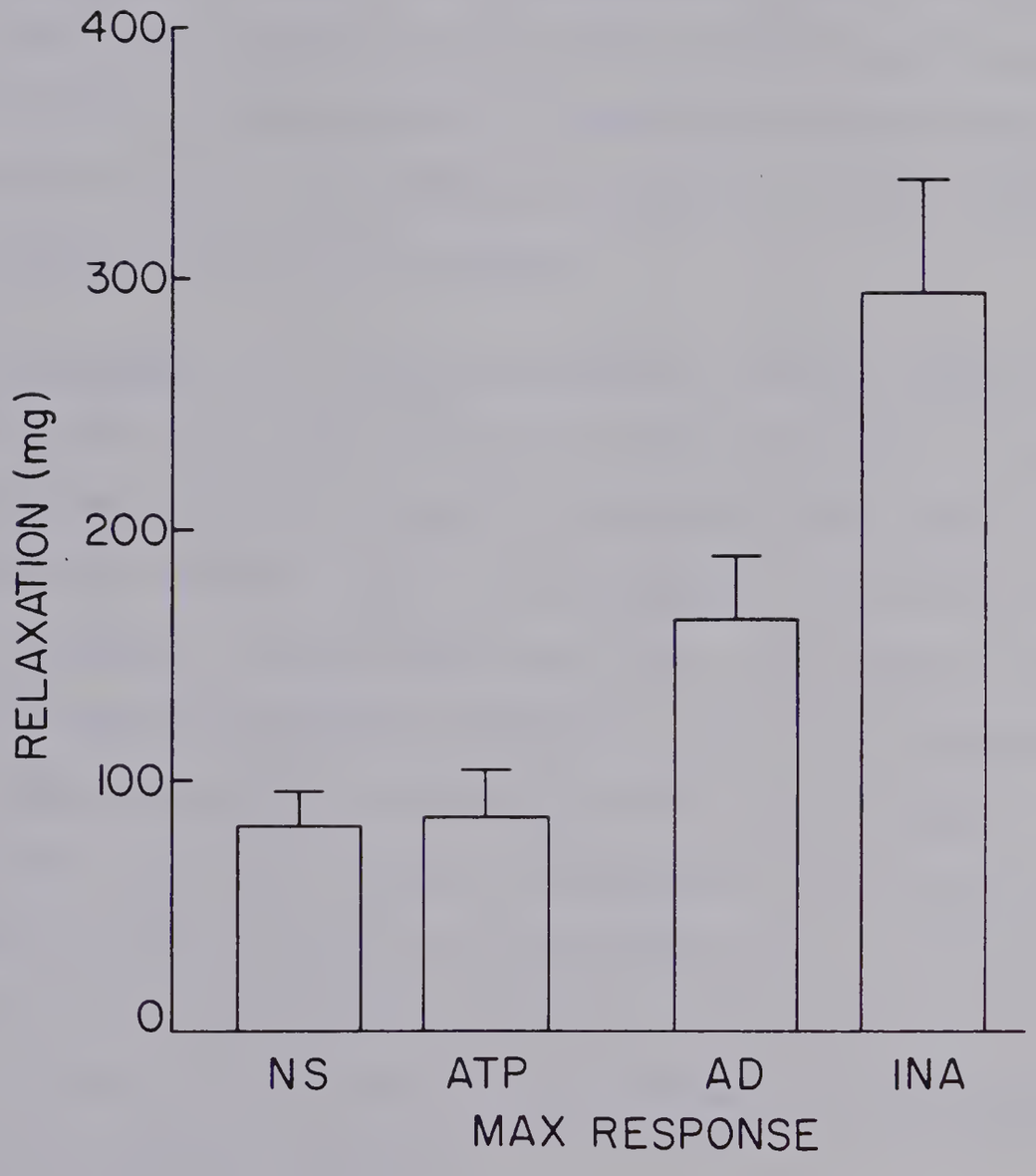

FIGURE 32

Histogram depicting maximum responses to NANCI field stimulation at 5 Hz (NS), ATP (10^{-3}M), adenosine (10^{-3}M AD), and isoproterenol (INA, 10^{-4}M). Mean values (n=5) are given; vertical lines show s.e. mean.

DISCUSSION

Summary of Results--The present study demonstrated a quantifiable relaxant effect of adenosine, and a dual action of ATP, on both guinea-pig and canine gallbladder. It also revealed a nonadrenergic, noncholinergic inhibitory nerve supply in both species; the mediator of this inhibitory response probably is not a purine compound.

Historical Background: The widespread inhibitory actions of adenosine and purine nucleotides (e.g., ATP), in particular the smooth-muscle relaxant effects, have been known for a long time. Drury and Szent-Györgyi (122) were the first to report the sedative, antispasmodic, and vasodilatory actions of adenosine. In the 50 years since then it has been shown that purine nucleosides and nucleotides have potent extracellular effects on excitable membranes, actions that may be involved in physiological processes (123). Particular attention has been paid to the actions of these compounds that could lead to clinical applications. Pre-eminent among these are the cardiovascular effects, that lead to vasodilation and hypotension (124), but the antilipolytic, antithrombic, and antispasmodic actions, also, have received attention.

Specific cells for the formation, storage, and release of other hormonal agents are known to exist but none has been identified for adenosine. Thus, in view of its widespread actions, adenosine may have a general regulatory function. A further role for purine compounds was suggested in 1972 when Burnstock (92) proposed the 'purinergic nerve'

hypothesis, but ATP rather than adenosine has been considered the neurotransmitter in these nerves.

ADENOSINE

Mechanism of action: Specific adenosine receptors that modulate adenylate cyclase activity have been identified on a wide variety of cell types. In 1972, Haslam and Lynham (125) showed that adenosine stimulates adenylate cyclase activity in platelets, which in turn increases the amount of cAMP. This concurs with the finding reported by Sattin and Rall (126) that adenosine increases the cAMP content in brain slices. However, cell-surface adenosine receptors that mediate decreases in cellular cAMP and adenylate cyclase activity have also been reported (127). In addition, an intracellular adenosine receptor site (P. site) which is inhibitory towards the action of adenylate cyclase has been found (128). No physiological role for this receptor (P.) has been established. On the basis of structure–activity studies with adenosine analogs, these different receptors on the surface of plasma cell membranes have been designated as A_2 (extracellular, cyclase-stimulating) and A_1 (extracellular, cyclase-inhibitory) by Van Calker, Muller, and Hamprecht (129). Londos and Wolff (130) referred to these adenosine receptor sites as R_i (cyclase inhibitory) and R_a (cyclase activating) while referring to the intracellular cyclase inhibitory site as P., indicating in this way that the R. site requires a relatively intact ribose moiety whereas the P. site tolerates ribose changes but no purine changes.

These receptors can be distinguished by selective analogs. Furthermore, agonist action at these sites can be antagonized by specific pharmacolo-

gical antagonists. The adenosine receptors (R_{a-i}) are analogous to the α and β-adrenergic receptors in some tissues.

In smooth-muscle preparations, however, there is evidence that adenosine does not act via the stimulation of adenylate cyclase. Schrader, Rubio, and Berne (131) and Herlihy et al. (132) showed that adenosine relaxes vascular smooth muscle by directly altering Ca^{++} ion permeability and/or membrane potential, a mechanism that does not support a role for cAMP in the adenosine-mediated response. Furthermore, McKenzie, Frew, and Baer (133) reported that, in the longitudinal muscle of rabbit small intestine, neither stimulation of adenylate cyclase nor changes in cAMP appeared to be part of the mechanism of smooth-muscle relaxation by adenosine or ATP. Despite continuing controversy over the role of adenylate cyclase in the action of adenosine in various tissues, the existence of extracellular adenosine and adenine nucleotide receptors is generally accepted.

Drug Modulation: A problem arises in assessing the effects of adenosine in tissues in that its apparent potency is masked by uptake and intracellular deamination (134, 135). It is obvious that quantification of the response to adenosine at its extracellular site requires prevention of its uptake into the cell.

Various pharmacological agents are known to inhibit the uptake of adenosine and, therefore, its metabolism to inactive products. Dipyridamole, which inhibits the transport of adenosine, has been widely used to demonstrate this effect (136, 137), but its lack of specificity (138) precludes the exact interpretation of its effects on nucleoside transport.

HNBTG was chosen for use in this study because of its more selective action as an inhibitor of nucleoside transport (139, 140).

Adenosine consistently mediated relaxation of intact guinea-pig gallbladder and isolated strip preparations of both guinea-pig and canine gallbladders. This response to adenosine in the gallbladder accords with the known inhibitory actions of adenosine in other tissues (141, 142). Furthermore, in the presence of the nucleoside-transport inhibitor, adenosine-mediated relaxations were significantly enhanced. This HNBTG-induced potentiation of the adenosine-mediated responses is further evidence that the adenosine receptors, like other hormone receptors, are situated on the external surface of the cell.

Various substances have been reported to inhibit the action of adenosine in certain tissues; these include the methylxanthine, 2-pyridyl-isatogen tosylate (PIT), and quinidine. As both quinidine and PIT probably are not specific, results obtained with these agents should be interpreted with caution; consequently, these agents were not used (143). Xanthine derivatives, theophylline, and caffeine (which show close structural resemblance to the purine compounds) have been reported to antagonize the pre- and postsynaptic effects of adenosine and/or ATP in gastrointestinal smooth muscle (144-146).

Aminophylline, which was used in the present study, is also known to inhibit cyclic nucleotide phosphodiesterase, an action that could explain its direct relaxant effect on smooth muscle when present in high concentrations (147). Therefore, a concentration was chosen that by itself did not have a direct effect on the tissue. Aminophylline significantly

antagonized adenosine-mediated relaxation in both the presence and absence of the adenosine-transport inhibitor HNBTG. When aminophylline was present, the shift of the adenosine-response curve was greater in HNBTG-treated tissues than in those in which adenosine uptake was not prevented. This is further evidence that the adenosine receptors are on the cell surface. The nature of this antagonism by aminophylline appears to be competitive; i.e., the slopes of the log concentration response curves in the presence of aminophylline were not significantly different from the controls, suggesting a parallel shift. These antagonistic actions of aminophylline on the adenosine-mediated responses in guinea-pig gallbladder are in agreement with previous findings in other tissues (141, 148).

ADENOSINE TRIPHOSPHATE (ATP)

ATP-mediated responses in the guinea-pig and dog gallbladders were more complex. The responses on untreated strip preparations were predominantly contractile; relaxations, and in some cases relaxation preceding a contraction, were seen occasionally with higher concentrations ($3X10^{-4}$ to $10^{-3}M$). However, only relaxant responses to ATP were seen when the background tone of the preparation was raised with CCK, and in tissues pretreated with the prostaglandin synthetase inhibitor indomethacin (120). Thus, it appears that ATP stimulates the production of prostaglandins that then mediate the contractile response. As the ATP-mediated relaxant responses were abolished by indomethacin, it seems that this response is independent of prostaglandin release. This dual action of ATP, in the guinea-pig and canine gallbladder, with the contractile phase being abolished by a prostaglandin synthetase inhibitor, is similar to that seen

in guinea-pig and rat stomach and ascending colon (149-151). Presumably, relaxant responses to ATP were not detected in the intact gallbladder because in this situation the synthesis of prostaglandins was not prevented and the background tone of the preparation was not raised with CCK.

Drug Modulation--Aminophylline and the adenosine transport inhibitor HNBTG had only weak if any effects on ATP-mediated relaxation. This is in contrast to the marked effects of both on adenosine-mediated relaxations. These findings suggest that adenosine and ATP act at different receptors, or by different mechanisms, to produce their characteristic responses. Alternatively, the relaxant response to ATP may be effected by adenosine which is produced by degradation of ATP. For example, ATP co-released with a neurotransmitter such as Ach can either act as specific ATP receptors or be hydrolysed to adenosine by ecto-ATPases or 5'-nucleotidase. Similarly, ATP may be released as the principal transmitter to act on receptors, or be hydrolysed to adenosine. In either case, the adenosine so formed could activate adenosine receptors. If a response to ATP requires hydrolysis of ATP to adenosine, the enzyme adenosine deaminase that converts adenosine into inosine (an inactive compound) will block or reduce this response by preventing the accumulation of adenosine.

The results in this study with adenosine deaminase showed that, in the gallbladder, a significant proportion of the relaxant response to ATP is due to the degradation of ATP to adenosine. However, adenosine deaminase did not abolish the relaxant response to ATP simultaneously with the abolition of the response to adenosine: this is evidence that ATP

can have a direct effect on the gallbladder.

Purinoceptors

Burnstock in 1978 (152) proposed that receptors for purine nucleotides could be classified into types P_1 and P_2. P_1 purinoceptors (which are equivalent to the R. receptor) are more sensitive to adenosine than ATP and are blocked by methylxanthines; occupation of this receptor leads to cAMP accumulation. P_2 purinoceptors are more sensitive to ATP than to adenosine, are blocked by apamin, and induce prostaglandin synthesis. It has also been proposed that P_2 receptors control ion fluxes and not adenylate cyclase activity (153).

However, apamin is not a specific antagonist, and in some cases (outlined above) adenosine does not act via adenylate cyclase. Therefore, the only deduction one can make with this system is that adenosine and ATP act at different receptor sites -- a finding that has been reported in many other tissues (154, 155).

Several attempts have been made to classify purinoceptors on biochemical or functional grounds. The problem with ATP, unlike adenosine, is that at present there appears to be no specific receptor antagonist. Characterization of its specific action requires stable analogs that are resistant to metabolism or uptake systems.

Adenosine Analogs

The structure—activity requirement of the adenosine-receptor system in guinea-pig gallbladders was probed with ligands that have been reported to distinguish between cyclase-associated adenosine receptor

subtypes (130).

2-Chloroadenosine, NECA, and PIA are analogs substituted at the 2, 6, and 5' positions of the purine ring. These analogs are not transported into cells or metabolized. The actions of these compounds were then compared with adenosine's, the latter being assayed in the presence of the adenosine-transport inhibitor HNBTG.

The responses to 2-chloroadenosine and adenosine in the presence of HNBTG possessed a similar relaxant potency. It has been reported that 2-chloroadenosine is 100 to 200 times more potent than adenosine in other tissues (156-158). This reportedly higher apparent potency of 2-chloroadenosine than of adenosine appears due to the former's being neither taken up into the tissue nor deaminated, and masking of adenosine's apparent potency by its rapid uptake and deamination (134, 135). In this study, when the uptake of adenosine was prevented with HNBTG, adenosine was as potent as 2-chloroadenosine. A similar potentiation of adenosine by HNBTG, in cardiac muscle, has been reported by Clanachan (159).

The low potency of PIA in this study may indicate R_a-type adenosine receptors in the gallbladder.

ELECTRICAL STIMULATION

Contractile Response: Field stimulation of both intact gallbladders and gallbladder strip preparations resulted in frequency-dependent contractile responses. These responses were abolished by prior treatment of the tissues with atropine, indicating that they had been mediated

via muscarinic receptors. These contractile responses were also antagonized by the sodium-channel blocker, TTX; this confirms that these responses were nerve-mediated. This confirms the findings in a previous report by Davison and Fösel (160) indicating that guinea-pig gallbladder has a cholinergic nerve supply. A similar cholinergic nerve supply was demonstrated in the dog gallbladder. Small contractile responses in the higher frequency ranges which were not antagonized by TTX probably indicate a direct effect on the muscle.

Relaxant Response: Field stimulation of the strip preparations following pre-treatment with adrenergic and cholinergic blocking agents (guanethidine and atropine) produced inhibitory responses. Guanethidine, an adrenergic-blocking agent is known to have direct anaesthetic effects on the tissue. However, in a series of experiments when guanethidine was replaced by the α and βadrenoceptor antagonists phentolamine and propranolol, similar relaxant responses on nerve stimulation were seen. These inhibitory responses were antagonized by TTX, indicating that they were nerve-mediated. These findings confirm the presence of nonadrenergic inhibitory responses in the guinea-pig gallbladder as reported by Davison and Fösel (160). In the present study, similar nonadrenergic noncholinergic inhibitory responses were demonstrated in the dog gallbladder and cystic duct.

Drug Modulation: The nerve-mediated inhibitory responses were potentiated in the lower frequency range (0.5 to 2 Hz) following pre-treatment of the tissue with the adenosine-transport inhibitor, HNBTG. Aminophylline produced a slight shift in the inhibitory response curve to field stimulation, relative to the control group. These findings are similar to

those seen with ATP but dissimilar to those obtained with adenosine.

Raised Tone: When the background tone of the preparation was raised with CCK, the amplitude of the inhibitory responses to field stimulation were increased in comparison with the control group. Surprisingly, inhibitory responses elicited in the presence of CCK were not antagonized by TTX. This may be explained by a number of possibilities:

(a) CCK could be interfering with the action of TTX.

(b) An impurity in CCK was either responsible for this effect or was antagonizing the effects of TTX.

(c) A population of TTX-resistant nerves were uncovered in the presence of raised tone.

(d) Direct electrical stimulation of the muscle was producing these effects.

Each of these points will now be discussed. Commercially available CCK is an impure preparation which contains other peptides; eg., motilin, which is known to cause relaxation of the gallbladder. In a further study (n=4), CCK was replaced by the synthetic octapeptide (Kinevac): however, TTX-resistant inhibitory responses were again demonstrated. It has also been reported that some agents [eg., trypsin (161)] can interfere with the action of TTX by splitting polypeptide chains on the carboxyl side of arginine or lysine residues. There is evidence that the TTX receptor contains one or the other of these amino acids. In order to exclude a similar action by CCK, histamine was used to raise the tone. However, TTX-resistant inhibitory responses were again demonstrated. Alternatively, these responses could be due to direct muscle stimulation. Reduction of the temperature of smooth-muscle preparations to 20° C interferes with

neuronal activity before causing any direct effects on the muscle (121). When the temperature of the preparation was reduced to 20° C, these inhibitory responses could not be demonstrated. The tissue was still viable as shown by its ability to contract after the addition of CCK and to relax in response to adenosine. These findings would thus suggest that these inhibitory responses were nerve-mediated and not due to a direct effect on the muscle.

METHOD OF STIMULATION

Alternatively, the method of stimulation, or the stimulation parameters used, could be responsible for these TTX-resistant inhibitory responses. It is known that stimulation of tissue in a longitudinal direction is more likely to cause direct muscle effects than stimulation in a transverse direction (121). This is due to two factors. One is the electrical anisotrophy of parallel-fibred muscle bundles; i.e., differences in tissue resistivities in the transverse and longitudinal directions. The ratio of transverse to longitudinal resistivities is about 10, so the current density for a given voltage through the tissue is less with a transverse field (162). The second factor is the greater number of cell membranes per unit length of tissue in the longitudinal than in the transverse direction because of the cell geometry (121).

In this study, inhibitory responses obtained after stimulation in a transverse direction were always abolished with TTX. TTX-resistant inhibitory responses were seen only with a longitudinally directed electric field. However, with longitudinal electrical field stimulation

when the pulse duration was reduced to 0.5 ms, and also making sure that the tissue was not in direct contact with the electrodes, TTX-sensitive inhibitory responses were demonstrated even when the background tone was raised with CCK. These findings indicate that transverse field stimulation is preferable to stimulation in a longitudinal direction. With longitudinal field stimulation, one must make sure that the tissue is not in contact with the electrodes. It is very unlikely that reducing the pulse duration to 0.5 ms had any significant effect, as it had no effect on the contractile responses, also the optimal pulse duration for direct muscle stimulation is 20-30 ms, not 0.5-1 ms (121).

NANCI NERVES

Nonadrenergic inhibitory responses were demonstrated in this study in both the guinea-pig and dog gallbladders and dog cystic duct. These inhibitory responses were seen in the lower frequency range, as has been reported for purinergic nerves in the other areas. These inhibitory responses were slightly potentiated by the adenosine-transport inhibitor HNBTG and similarly partly antagonized by aminophylline. These results are the opposite to those seen with adenosine but bear a resemblance to those seen with ATP. However, the purinergic-nerve hypothesis proposes that ATP, rather than adenosine, is the mediator of these responses. It was evident from these results that further studies were needed to investigate the possible role of adenosine as the mediator of these responses.

Autoinhibition: In some tissues, following their continual exposure to a high level of an agonist a change occurs in their functional activity. This

change is manifested by a loss in the sensitivity of the tissue to the action of that agent. The mechanism of action of this process is probably related to receptor modulation (163, 164). If one could show cross desensitization between ATP or adenosine and the nerve-mediated response it would be very strong evidence in favour of one of these substances as being the neurotransmitter involved in nonadrenergic noncholinergic inhibitory responses.

In the case of adenosine and ATP, actual tachyphylaxis does not occur; i.e., after exposure of the tissues to these agents then washing the tissue, responses are maintained following their reintroduction. However, in the continual presence of these purines, the responses of some tissues fade while still responding fully to agonists such as noradrenaline, although remaining refractory to adenosine/ATP or other agents acting on the same receptor. This phenomenon therefore was called autoinhibition.

It was shown in this study that dog gallbladder strips also show fade of relaxant responses (i.e., autoinhibition) to adenosine and ATP after their continual application to the tissue. In tissues autoinhibited to adenosine or ATP, field stimulation still produced inhibitory responses which were not reduced in magnitude compared with the inhibitory responses obtained before autoinhibition. This result means that even when all the ATP and adenosine receptor sites were either occupied or modulated, the inhibitory transmitter released in response to field stimulation was still capable of eliciting maximum responses. This finding is very strong evidence that neither ATP nor adenosine is the mediator of these inhibitory responses.

During these studies, it was noted that the potency of adenosine and ATP was low. This observation has been noted in the past by other authors in preparations supplied with nonadrenergic noncholinergic inhibitory nerves (NANCI); e.g., guinea-pig trachea (165) and urinary bladder (166). However, if ATP or adenosine is the neurotransmitter of NANCI nerves in the guinea-pig or dog gallbladder, it would appear that a high local concentration, perhaps in the order of 10^{-4}M, must be attained following stimulation of these nerves.

If ATP or adenosine is not the mediator of these inhibitory responses, then what is?

Several other agents (e.g., VIP, somatostatin, 5 HT) have been proposed in the past (92, 96). All hormones thus far isolated from the gastrointestinal tract are peptides, so that it would seem likely that the mediator of this response is also a peptide. VIP would seem a likely candidate as it is known to cause relaxation of the gallbladder (516-517) and nerves containing VIP have been located in the wall of the gallbladder (62).

PHYSIOLOGICAL ROLE OF NANCI NERVES

Because these nonadrenergic, noncholinergic inhibitory responses were demonstrated in two species and in the dog cystic duct they may have a role to play in the control of gallbladder motility. To quantitate these roles during the storage phase of the gallbladder, an *in vivo* study to determine pressure/volume curves for gallbladder filling before and after denervation

of the gallbladder should be performed. It is difficult to see how stimulation of these nonadrenergic noncholinergic inhibitory fibres alone could mediate receptive relaxation, as the maximum responses obtained after their stimulation was only 28.8% of the maximum β-adrenoceptor stimulation. However, it may well be that stimulation of these NANCI nerves in conjunction with sympathetic-nerve stimulation may play a major role in the control of gallbladder motility during its storage phase.

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ABSTRACTS

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